

World Journal of Gastroenterology®

Volume 13 Number 36
September 28, 2007



National Journal Award
2005



Editorial Department of *World Journal of Gastroenterology*
77 Shuangta Xijie, Taiyuan 030001, Shanxi Province, China
Telephone: +86-351-4078656
E-mail: wjg@wjgnet.com
<http://www.wjgnet.com>

ISSN 1007-9327 CN 14-1219/R Local Post Offices Code No. 82-261

World Journal of Gastroenterology

www.wjgnet.com

Volume 13

Number 36

Sep 28

2007



ISSN 1007-9327
CN 14-1219/R



WJG

World Journal of Gastroenterology®

Indexed and Abstracted in:

Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®) and Journal Citation Reports/Science Edition, *Index Medicus*, MEDLINE and PubMed, Chemical Abstracts, EMBASE/Excerpta Medica, Abstracts Journals, *Nature Clinical Practice Gastroenterology and Hepatology*, CAB Abstracts and Global Health.
ISI JCR 2003-2000 IF: 3.318, 2.532, 1.445 and 0.993.

Volume 13 Number 36 September 28, 2007

World J Gastroenterol
2007 September 28; 13(36): 4791-4916

Online Submissions

wjg.wjgnet.com
www.wjgnet.com

Printed on Acid-free Paper

世界胃肠病学杂志

A Weekly Journal of Gastroenterology and Hepatology



National Journal Award
2005

World Journal of Gastroenterology®

Weekly Established in October 1995

Volume 13 Number 36
September 28, 2007



Contents

EDITORIAL

- 4791 Transient elastography for the assessment of chronic liver disease: Ready for the clinic?
Cobbold JFL, Morin S, Taylor-Robinson SD
- 4798 Stimulating erythropoiesis in inflammatory bowel disease associated anemia
Tsiolakidou G, Koutroubakis IE

TOPIC HIGHLIGHT

- 4807 Immunity to hepatitis C virus infection: Update 2007
Thimme R
- 4808 Sequence diversity of hepatitis C virus: Implications for immune control and therapy
Timm J, Roggendorf M
- 4818 Interaction of hepatitis C virus with the type I interferon system
Weber F
- 4824 Neutralizing antibodies in hepatitis C virus infection
Zeisel MB, Fafi-Kremer S, Fofana I, Barth H, Stoll-Keller F, Doffoël M, Baumert TF
- 4831 CD4+ T cell responses in hepatitis C virus infection
Semmo N, Klenerman P
- 4839 Host and viral factors contributing to CD8+ T cell failure in hepatitis C virus infection
Neumann-Haefelin C, Spangenberg HC, Blum HE, Thimme R
- 4848 Memory CD8+ T cell differentiation in viral infection: A cell for all seasons
Radziejewicz H, Uebelhoer L, Bengsch B, Grakoui A
- 4858 Regulatory T cells in viral hepatitis
Billerbeck E, Böttler T, Thimme R
- 4865 Hepatitis C virus infection and apoptosis
Fischer R, Baumert T, Blum HE

BASIC RESEARCH

- 4873 Effects and mechanisms of electroacupuncture at PC6 on frequency of transient lower esophageal sphincter relaxation in cats
Wang C, Zhou DF, Shuai XW, Liu JX, Xie PY

CLINICAL RESEARCH

- 4881 Impaired contractility and remodeling of the upper gastrointestinal tract in diabetes mellitus type-1
Frøkjær JB, Andersen SD, Ejlskjær N, Funch-Jensen P, Drewes AM, Gregersen H

- 4891** Ferucarbotran *versus* Gd-DTPA-enhanced MR imaging in the detection of focal hepatic lesions
Cheng WZ, Zeng MS, Yan FH, Rao SX, Shen JZ, Chen CZ, Zhang SJ, Shi WB
- 4897** Endoscopic management of gastrointestinal smooth muscle tumor
Zhou XD, Lv NH, Chen HX, Wang CW, Zhu X, Xu P, Chen YX

- RAPID COMMUNICATION 4903** Does protracted antiviral therapy impact on HCV-related liver cirrhosis progression?
Tarantino G, Gentile A, Capone D, Basile V, Tarantino M, Di Minno MND, Cuocolo A, Conca P

- CASE REPORT 4909** Secondary pancreatic involvement by a diffuse large B-cell lymphoma presenting as acute pancreatitis
Saif MW, Khubchandani S, Walczak M

- ACKNOWLEDGMENTS 4912** Acknowledgments to Reviewers of *World Journal of Gastroenterology*

- APPENDIX 4913** Meetings
- 4914** Instructions to authors

- FLYLEAF I-V** Editorial Board

- INSIDE FRONT COVER** Online Submissions

- INSIDE BACK COVER** Online Submissions

Responsible E-Editor for this issue: Wen-Hua Ma

C-Editor for this issue: Gianfranco Alpini, PhD, Professor

Responsible S-Editor for this issue: Ye Liu

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*), a leading international journal in gastroenterology and hepatology, has an established reputation for publishing first class research on esophageal cancer, gastric cancer, liver cancer, viral hepatitis, colorectal cancer, and *H pylori* infection, providing a forum for both clinicians and scientists, and has been indexed and abstracted in Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®) and Journal Citation Reports/Science Edition, *Index Medicus*, MEDLINE and PubMed, Chemical Abstracts, EMBASE/Excerpta Medica, Abstracts Journals, *Nature Clinical Practice Gastroenterology and Hepatology*, CAB Abstracts and Global Health. ISI JCR 2003-2000 IF: 3.318, 2.532, 1.445 and 0.993. *WJG* is a weekly journal published by *WJG*. The publication date is on 7th, 14th, 21st, and 28th every month. The *WJG* is supported by The National Natural Science Foundation of China, No. 30224801 and No.30424812, which was founded with a name of *China National Journal of New Gastroenterology* on October 1, 1995, and renamed as *WJG* on January 25, 1998.

NAME OF JOURNAL
World Journal of Gastroenterology

RESPONSIBLE INSTITUTION
Department of Science and Technology
of Shanxi Province

SPONSOR
Taiyuan Research and Treatment Center
for Digestive Diseases, Taiyuan 77,
Shuangta Xijie, Taiyuan 030001, Shanxi
Province, China

EDITING
Editorial Board of *World Journal of
Gastroenterology*, 77 Shuangta Xijie,
Taiyuan 030001,
Shanxi Province, China
Telephone: +86-351-4078656
E-mail: wjg@wjgnet.com

PUBLISHING
Editorial Department of *World Journal
of Gastroenterology*, 77 Shuangta Xijie,
Taiyuan 030001,
Shanxi Province, China
Telephone: +86-351-4078656
E-mail: wjg@wjgnet.com
http://www.wjgnet.com

PRINTING
Beijing Kexin Printing House

OVERSEAS DISTRIBUTOR
Beijing Bureau for Distribution of
Newspapers and Journals
(Code No. 82-261)
China International Book Trading
Corporation PO Box 399, Beijing,
China (Code No. M4481)

PUBLICATION DATE
September 28, 2007

EDITOR-IN-CHIEF
Lian-Sheng Ma, Taiyuan

SUBSCRIPTION
RMB 50 Yuan for each issue, RMB 2400
Yuan for one year

CSSN
ISSN 1007-9327
CN 14-1219/R

HONORARY EDITORS-IN-CHIEF

Ke-Ji Chen, *Beijing*
Li-Fang Chou, *Taipei*
Zhi-Qiang Huang, *Beijing*
Shinn-Jang Hwang, *Taipei*
Min-Liang Kuo, *Taipei*
Nicholas F LaRusso, *Rochester*
Jie-Shou Li, *Nanjing*
Geng-Tao Liu, *Beijing*
Lein-Ray Mo, *Tainan*
Bo-Rong Pan, *Xi'an*
Fa-Zu Qiu, *Wuhan*
Eamonn M Quigley, *Cork*
David S Rampton, *London*
Rudi Schmid, *kentfield*
Nicholas J Talley, *Rochester*
Guido NJ Tytgat, *Amsterdam*
H-P Wang, *Taipei*
Jaw-Ching Wu, *Taipei*
Meng-Chao Wu, *Shanghai*
Ming-Shiang Wu, *Taipei*
Jia-Yu Xu, *Shanghai*
Ta-Sen Yeh, *Taiyuan*

ASSOCIATE EDITORS-IN-CHIEF

Gianfranco D Alpini, *Temple*
Bruno Annibale, *Roma*
Roger William Chapman, *Oxford*
Chi-Hin Cho, *Hong Kong*
Alexander L Gerbes, *Munich*
Shou-Dong Lee, *Taipei*
Walter Edwin Longo, *New Haven*

You-Yong Lu, *Beijing*
Masao Omata, *Tokyo*
Harry HX Xia, *Hanover*

SCIENCE EDITORS
Deputy Director: Ye Liu, *Beijing*
Jian-Zhong Zhang, *Beijing*

LANGUAGE EDITORS
Director: Jing-Yun Ma, *Beijing*
Deputy Director: Xian-Lin Wang, *Beijing*

MEMBERS
Gianfranco D Alpini, *Temple*
BS Anand, *Houston*
Richard B Banati, *Lidcombe*
Giuseppe Chiarioni, *Vareggio*
John Frank Di Mari, *Texas*
Shannon S Glaser, *Temple*
Mario Guslandi, *Milano*
Martin Hennenberg, *Bonn*
Atif Iqbal, *Omaha*
Manoj Kumar, *Nepal*
Patricia F Lalor, *Birmingham*
Ming Li, *New Orleans*
Margaret Lutze, *Chicago*
Jing-Yun Ma, *Beijing*
Daniel Markovich, *Brisbane*
Sabine Mihm, *Göttingen*
Francesco Negro, *Genève*
Bernardino Rampone, *Siena*
Richard A Rippe, *Chapel Hill*
Stephen E Roberts, *Swansea*
Ross C Smith, *Sydney*
Seng-Lai Tan, *Seattle*
Xian-Lin Wang, *Beijing*
Eddie Wisse, *Keerbergen*
Daniel Lindsay Worthley, *Bedford*

COPY EDITORS
Gianfranco D Alpini, *Temple*
Sujit Kumar Bhattacharya, *Kolkata*
Filip Braet, *Sydney*
Kirsteen N Browning, *Baton Rouge*

Radha K Dhiman, *Chandigarh*
John Frank Di Mari, *Texas*
Shannon S Glaser, *Temple*
Martin Hennenberg, *Bonn*
Eberhard Hildt, *Berlin*
Patricia F Lalor, *Birmingham*
Ming Li, *New Orleans*
Margaret Lutze, *Chicago*
MI Torres, *Jain*
Sri Prakash Misra, *Allahabad*
Giovanni Monteleone, *Rome*
Giovanni Musso, *Torino*
Valerio Nobili, *Rome*
Osman Cavit Ozdogan, *Istanbul*
Francesco Perri, *San Giovanni Rotondo*
Thierry Piche, *Nice*
Bernardino Rampone, *Siena*
Richard A Rippe, *Chapel Hill*
Ross C Smith, *Sydney*
Daniel Lindsay Worthley, *Bedford*
George Y Wu, *Farmington*
Jian Wu, *Sacramento*

COPYRIGHT
© 2007 Published by *WJG*. All rights
reserved; no part of this publication
may be reproduced, stored in a retrieval
system, or transmitted in any form or
by any means, electronic, mechanical,
photocopying, recording, or otherwise
without the prior permission of *WJG*.
Authors are required to grant *WJG* an
exclusive licence to publish.

SPECIAL STATEMENT
All articles published in this journal
represent the viewpoints of the authors
except where indicated otherwise.

INSTRUCTIONS TO AUTHORS
Full instructions are available online at
http://www.wjgnet.com/wjg/help/
instructions.jsp. If you do not have web
access please contact the editorial office.



Transient elastography for the assessment of chronic liver disease: Ready for the clinic?

JFL Cobbold, S Morin, SD Taylor-Robinson

JFL Cobbold, S Morin, SD Taylor-Robinson, Division of Medicine, Faculty of Medicine, Imperial College London, London, United Kingdom

Supported by a Centenary Fellowship from the Hammersmith Hospital Trustees Research Committee, London, United Kingdom; The British Medical Research Council (G99000178); The United Kingdom Engineering Physics and Science Research Council, Pfizer Global Research Ltd, Sandwich, United Kingdom; and The United Kingdom Department of Health Research and Development Fund

Correspondence to: Dr. JFL Cobbold, Robert Steiner MRI Unit, Imperial College London, Hammersmith Hospital Campus, DuCane Road, W12 0HS London,

United Kingdom. j.cobbold@imperial.ac.uk

Telephone: +44-20-83835856 Fax: +44-20-83833038

Received: June 27, 2007 Revised: July 9, 2007

Abstract

Transient elastography is a recently developed non-invasive technique for the assessment of hepatic fibrosis. The technique has been subject to rigorous evaluation in a number of studies in patients with chronic liver disease of varying aetiology. Transient elastography has been compared with histological assessment of percutaneous liver biopsy, with high sensitivity and specificity for the diagnosis of cirrhosis, and has also been used to assess pre-cirrhotic disease. However, the cut-off values between different histological stages vary substantially in different studies, patient groups and aetiology of liver disease. More recent studies have examined the possible place of transient elastography in clinical practice, including risk stratification for the development of complications of cirrhosis. This review describes the technique of transient elastography and discusses the interpretation of recent studies, emphasizing its applicability in the clinical setting.

© 2007 WJG. All rights reserved.

Key words: FibroScan; Transient elastography; Liver stiffness measurement; Hepatic fibrosis; Hepatitis

Cobbold JFL, Morin S, Taylor-Robinson SD. Transient elastography for the assessment of chronic liver disease: Ready for the clinic? *World J Gastroenterol* 2007; 13(36): 4791-4797

<http://www.wjgnet.com/1007-9327/13/4791.asp>

INTRODUCTION

The management and prognosis of chronic liver disease is strongly influenced by its severity. While percutaneous liver biopsy remains the gold standard, there is increasing awareness, not only of the associated morbidity and mortality of the procedure, but also its diagnostic limitations. There is considerable sampling variability, and inter- and intra-observer variation in the assessment of liver pathology. Antifibrotic therapies are in development, but it has been stated that “the lack of robust markers of fibrosis represents the single greatest factor limiting both the validation of progression or regression of fibrosis and the testing of antifibrotic therapies”^[1].

A number of approaches to non-invasive assessment of chronic liver disease have been developed. Serum markers, and serum panel markers for the assessment of chronic liver disease, such as the APRI (AST to platelet ratio index) score, Enhanced (European) Liver Fibrosis (ELFTM) test and FibroTest, have been proposed, and are the subject of several comprehensive reviews^[2-4]. Investigations based on imaging modalities, including ultrasound and magnetic resonance, are liver-specific and provide structural information related to the liver^[5]. Microbubble contrast-enhanced ultrasound to obtain hepatic vein transit times (HVTT) and phosphorus-31 magnetic resonance spectroscopy (³¹P MRS) have been shown to delineate cirrhotic and pre-cirrhotic disease stages, but require considerable operator skill and access to the relevant technology^[6-10]. Other MR techniques, such as diffusion-weighted imaging (DWI) and ultrashort echotime (UTE) have shown promise, but require further development^[11,12]. Moreover, these techniques require assessment in larger subject groups in the setting of multi-centre trials.

Liver stiffness measurement using transient elastography (TE) (FibroScan[®]) is a recently developed technique designed for the assessment of liver fibrosis, and has been extensively evaluated in several recent studies. The aim of this article is to review the current data on the use of transient elastography in clinical practice and to make recommendations for future research. A Medline search using the terms “FibroScan” and “transient elastography” was conducted. The proceedings of the 41st annual meeting of the European Association for the Study of the Liver 2006, and the 57th annual meeting of the American Association for the Study of Liver Diseases 2006 were also searched for relevant articles.

THE BASIS OF TRANSIENT ELASTOGRAPHY

Transient elastography allows liver stiffness measurement (LSM) which enables the assessment of liver disease severity, using a 1-dimensional ultrasound transducer and receiver mounted on the same axis as a vibrator, producing a low-frequency pulse or shear wave. When the probe tip is placed perpendicularly against the skin between the ribs overlying the liver and triggered, the rate of progression of the shear wave is measured.

The speed of propagation depends on the elasticity or stiffness of the tissue under examination and is measured by a series of ultrasonic pulses, which detect the transient local deformations in the liver tissue as the shear wave progresses. The elasticity of the liver is derived from the velocity of the wave approximating to the Young's modulus, E , according to the equation: $E = 3\rho V_s^2$, where V_s is the shear velocity and ρ is the mass density, assumed to be close to that of water. The deformation of tissue is plotted as a function of time and depth to create a two-dimensional "elastogram". The slope of the elastogram represents the speed of propagation and thus the liver stiffness, expressed in kPa^[13].

This technique is simple to learn, can be performed quickly by a single operator, and provides an objective measure of liver stiffness. TE has been employed in a number of clinical paradigms over the last few years, however, there is at present no consensus on its indications for use, interpretation and applicability.

ANALYSIS AND EVALUATION

Most studies carried out to evaluate the performance of transient elastography compare LSM to the histological assessment of liver biopsy. Of these, the METAVIR scoring system which is widely used in clinical practice has been employed in the majority of studies^[14]. Fibrosis is staged semi-quantitatively on a five-point scale from F0 to F4 (F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis and few septa; F3 numerous septa without cirrhosis; F4, cirrhosis). Divisions of clinical significance are considered to be between F1 and F2 (from minimal to significant fibrosis) and between F3 and F4 (from fibrosis to cirrhosis). The data obtained is commonly represented by boxplots with liver stiffness (or log₁₀ liver stiffness) on the y axis and fibrosis stage on the x axis, representing the median, interquartile range and range of values for individuals at each fibrosis stage. Despite an apparently high diagnostic accuracy, there is substantial overlap between groups, especially in the pre-cirrhotic stage of liver disease. This has clinical implications since for a given liver stiffness measurement, the patient's true fibrosis score may vary from F0-1 to F4^[15].

A major criticism of liver biopsy and an important stimulus to the development of non-invasive techniques is the small portion of liver that is assessed. The specimen obtained by standard liver biopsy techniques represents just 1/50 000 of the liver, and typically only 16% of such biopsies exceed the optimal length of 25 mm required

for adequate histological assessment^[16]. This results in significant sampling variability since hepatic fibrosis, inflammation and steatosis may have a patchy spatial distribution within the liver. Added to this drawback is the presence of inter- and intra- observer variability in histological assessment, making this "gold-standard" considerably flawed. Thus, non-invasive measures such as TE are judged by liver biopsy which may be a flawed standard. Clearly, the results obtained should be regarded as a probability of correctly predicting liver fibrosis and interpreted in the context of clinical, epidemiological and biochemical data, and moreover should be correlated prospectively with robust clinical outcome measures.

ASSESSMENT OF DIAGNOSTIC ACCURACY

While the sensitivity, specificity, positive and negative predictive values describe the performance of a test with respect to a gold-standard, diagnostic accuracy provides a measure of the overall performance of a test. Diagnostic accuracy of transient elastography with respect to histology has been measured in clinical studies using the area under the receiver operator characteristic (ROC) curve, a convenient non-parametric method for the assessment of diagnostic tests, compared to a gold-standard. The sensitivity is plotted against 1- specificity for all possible cut-off values between two states. For example, this could be expressed as: positive or negative; cirrhosis or no cirrhosis; insignificant or significant fibrosis. A measure of diagnostic accuracy of the test may be derived, whereby an area near 1 represents high diagnostic accuracy. ROCs may be used to select cut-off values appropriate to different scenarios. Cut-off values may then be selected for a given situation according to the required sensitivity or specificity of a given value to distinguish the two states. This allows cut-off values to be chosen to answer clinically relevant questions. For example, to rule out cirrhosis effectively in a group of patients, a cut-off value with a specificity of 95% may be used, indicating that there is a 95% probability that patients below the cut-off value will not have cirrhosis. Alternatively, an optimum cut-off may be calculated, where the cut-off is chosen at the point where the sum of sensitivity and specificity is maximal, although this is affected by the shape of the ROC curve and therefore may vary between studies. A more inclusive measure would be obtained by using a cut-off associated with high sensitivity but lower specificity.

The largest study published to date demonstrates that different cut-off values for the diagnosis of cirrhosis exist, depending on the sensitivity and specificity required for the decision and for different aetiologies, such as chronic hepatitis B, chronic hepatitis C and alcohol-related and non-alcoholic related fatty liver disease (Table 1)^[17].

TRANSIENT ELASTOGRAPHY FOR STAGING OF HEPATIC FIBROSIS

Initial clinical studies using TE investigated the ability of

Table 1 Liver stiffness cut-off values for the diagnosis of cirrhosis according to the primary cause of liver disease

	Optimum cutoff (kPa)		
	Hepatitis C (<i>n</i> = 298)	Hepatitis B (<i>n</i> = 122)	Alcohol or NASH (<i>n</i> = 122)
Sensitivity 95%	10.0	6.0	13.2
Max. sum of sensitivity and specificity	10.4	10.3	21.5
Best diagnostic accuracy	20.2	16.9	21.5
Specificity 95%	14.1	14.3	27.7

NASH: Non-alcoholic steatohepatitis. Data obtained from Ganne-Carrie *et al* 2006^[17].

the technique to assess hepatic fibrosis when compared to the gold standard of liver biopsy. This was assessed by Sandrin and colleagues in a “proof-of-principle” study. They demonstrated a graduated increase in liver stiffness with increasing hepatic fibrosis^[13]. Chronic hepatitis C was chosen as the paradigm for many studies as the patients are numerous and the natural history of the disease and histological classification systems have been well described^[14,18]. Other workers have investigated the ability of TE to assess fibrosis compared to liver biopsy in several disease paradigms, as summarised in Table 2^[13,15,17,19-25].

Hepatic fibrosis is a complex and multistep process. Therefore, a precise description of disease severity may require assessment of more than one aspect of the disorder. TE measures liver stiffness which is thought to be due largely to the extent of fibrosis. Indeed digital image analysis demonstrates a correlation between the fibrotic area and liver stiffness^[26]. Yet, the extent of fibrosis does not provide the complete picture. Histological scoring systems are not linear and in addition to the extent, they describe the pattern of deposition of fibrous tissue^[18,14]. The effect of collagen cross-linkage on liver stiffness, associated with more severe disease, has not been clearly established. More recently, a strong relationship between liver stiffness and the hepatic venous pressure gradient (HVPG) has been described, demonstrating an association with portal hypertension^[27]. The relative contribution of fibrosis, inflammation and haemodynamic changes have yet to be determined.

A recent well-conducted study by Fraquelli and colleagues looked specifically at the reproducibility of LSM in 195 patients with liver disease of mixed aetiology (predominantly HCV)^[24]. The results obtained by two different operators working under highly regulated conditions, showed a very high degree of inter-observer agreement, with an intraclass correlation coefficient (ICC) of 0.98, representing an estimated 98% of variability due to patient characteristics, as opposed to observer variability. This agreement was substantially reduced when groups such as overweight patients, those with histological or ultrasound evidence of hepatic steatosis and especially those with mild disease (ICC 0.6 for METAVIR F0-1 *vs* 0.99 for F ≥ 2) were assessed. There were stringent inclusion criteria such as inclusion of only those patients in whom a success rate of > 65% was achieved and where the

interquartile range of the readings was less than 30% of the median. Additionally, 76% of liver biopsy specimens exceeded 20 mm in length, thus minimising sampling error. Despite the high reproducibility of LSM, there was substantial overlap in the findings between adjacent stages of hepatic fibrosis, which the authors acknowledged would limit the diagnostic accuracy of TE, particularly in intermediate fibrosis stages.

Disease activity or necro-inflammation are not directly assessed by TE, although LSM has been shown to increase with increasing necroinflammatory scores at histology^[24], and in biochemically-assessed flares of hepatitis and cirrhosis^[28]. Steatosis is a cause as well as a consequence of chronic liver disease^[29], and its effect on liver stiffness is believed to be minimal based on multivariate analyses in studies investigating other endpoints^[15,30]. However, studies assessing the severity of liver stiffness stratified by the degree of steatosis are awaited. The development of serum panel markers of hepatic fibrosis demonstrates recognition of the fact that multiple diagnostic measures are required. Castera and colleagues compared TE with FibroTest, an indirect serum panel marker, and the AST to platelet ratio index (APRI) in a cohort of patients with chronic hepatitis C, and found equivalent results with these techniques, but noted that a combination of TE and the serum panel marker provided the greatest diagnostic accuracy^[19]. On this basis, an algorithm was proposed whereby liver biopsy can be avoided in most patients with chronic hepatitis C when the tests are in agreement^[19]. Other studies have demonstrated a similar diagnostic accuracy of TE in the context of hepatitis C virus (HCV) and human immunodeficiency virus (HIV) coinfecting patients^[23], and also in the presence of biliary fibrosis in patients with primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC)^[20,23].

CIRRHOSIS AND ITS COMPLICATIONS

In view of the wide range of LSM for any single fibrosis stage, attention has turned to the use of FibroScan in the diagnosis of cirrhosis and of its complications such as varices, risk of variceal bleeding, hepatocellular carcinoma and ascites. Foucher and colleagues examined prospectively a cohort of patients with liver disease of varying aetiology^[25]. In addition to establishing the cut-off values for fibrosis of varying severity as assessed by liver biopsy, they calculated the cut-off values below which there was a 90% chance that complications such as varices (stage 2/3) (27.5 kPa), Child-Pugh score of B/C (37.5 kPa), ascites (49.1 kPa), and oesophageal bleeding (62.7 kPa) were absent. Although the number of subjects with each complication was small (between 14 and 42 cases), these cut-off values may serve to identify patients with cirrhosis at risk of such complications. However, history of ascites, hepatocellular carcinoma, and variceal bleeding may be readily obtained by direct questioning of the patient, therefore for these observations to be clinically useful, they need to be borne out in prospective studies on the development of these complications over time. Kazemi and colleagues correlated endoscopic evidence of oesophageal varices with LSM in a cohort of patients with

Table 2 Results of studies in which liver stiffness was compared with histological fibrosis stage (METAVIR system) to establish diagnostic accuracy and cut-off values

Author, yr	Patient group	Number of subjects	Cut-off for $F \geq 2$ (kPa)	AUROC $F \geq 2$	Cut-off for $F = 4$ (kPa)	AUROC $F = 4$
Fraquelli M <i>et al</i> 2007 ^[24]	Mixed (HCV)	195 (155)	7.9 (72%; 84%)	0.86	11.9 (91%; 89%)	0.90
Ganne-Carrie N <i>et al</i> 2006 ^[17]	Mixed (HCV)	1007 (298)			14.6 (79%; 95%)	0.92
De Ledinghen V <i>et al</i> 2006 ^[23]	HCV/HIV co-infected	72	4.5 (93%; 17%)	0.72	11.8 (100%; 93%)	0.97
Gomez-Dominguez E <i>et al</i> 2006 ^[22]	Mixed (HCV)	94 (62)	4 (94%; 33%)	0.74	16 (89%; 96%)	0.94
Carrión J <i>et al</i> 2006 ^[21]	HCV post transplant	169	8.5 (90%; 81%)	0.90	12.5 (100%; 87%)	0.98
Corpechot C <i>et al</i> 2006 ^[20]	PBC/PSC	101	7.3 (84%; 87%)	0.92	17.3 (93%; 95%)	0.96
Foucher J <i>et al</i> 2006 ^[25]	Mixed	354	7.2 (64%; 85%)	0.80	17.6 (77%; 97%)	0.96
Castera L <i>et al</i> 2005 ^[31]	HCV	183	7.1 (67%; 89%)	0.83	12.5 (87%; 91%)	0.95
Ziol M <i>et al</i> 2005 ^[15]	HCV	251	8.8 (56%; 91%)	0.79	14.6 (86%; 96%)	0.97
Sandrin <i>et al</i> 2003 ^[13]	HCV	106		0.88		0.99

Cut-off values were those proposed by the authors. If more than one cut-off value was available, the value set for optimum diagnostic accuracy (i.e. cut-off at which sensitivity (se) + specificity (sp) is maximal) was used. Cut-off values are followed by the relevant sensitivity and specificity in parenthesis. HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; PSC: Primary sclerosing cholangitis; PBC: Primary biliary cirrhosis.

compensated cirrhosis, and observed that a cut-off value of 19 kPa predicted varices (stage II / III) with a sensitivity of 91% and specificity of 60%^[31]. These findings suggest that endoscopic surveillance can be avoided in up to 60% patients with cirrhosis.

TOWARDS THE CLINIC

Ganne-Carrie and coworkers have published the largest series to date, but have concentrated on separation of cirrhotic from precirrhotic disease^[17]. These authors highlighted the fact that the cut-off values vary on the basis of the aetiology of liver disease as well as the level of sensitivity and specificity required for the issue under question (Table 1). There were substantial differences in the cut-off values for the prediction of cirrhosis with 95% specificity depending on the number of patients studied (775 *vs* 1007). It remains to be seen whether the cut-off values vary depending on the population of interest, for example in affluent societies with high level of co-existent type 2 diabetes and hepatic steatosis, compared to a group with few risk factors for steatosis.

Of particular interest is the question whether liver stiffness measurement affects clinical decision making. For example, a non-invasive technique, such as TE, is particularly desirable in patients with bleeding disorders, such as haemophilia. Two studies have addressed this issue. Masaki and colleagues correlated LSM with ultrasound assessment of liver disease and several serum markers in haemophiliacs coinfecting with HCV and HIV, although the findings were not linked to a clear standard or to clinical outcome measures and decisions^[32]. Posthouwer and colleagues studied a cohort of patients with haemophilia and chronic hepatitis C in whom percutaneous liver biopsy was contraindicated^[33]. Cut-off values from a previous study were applied^[19] and validated in a separate cohort of patients without a bleeding disorder. This study demonstrated the pragmatic use of TE in a specific scenario with acknowledgement that there will be inaccurate assessment in a proportion of patients. In such a scenario, a false positive test may result in a patient with mild disease receiving antiviral therapy while a patient

with a false negative test, may have severe disease and not receive treatment. It can be argued that the latter group may have a detrimental outcome, and therefore a cut-off with a higher sensitivity is indicated.

An important indication for non-invasive staging of hepatitis C-related liver disease is to determine whether antiviral treatment is appropriate in a particular patient^[13]. However, on the basis of recent studies in patients with mild hepatitis C, current UK treatment guidelines suggest that treatment is both clinically effective and cost-effective in histologically mild, moderate and severe stages of pre-cirrhotic disease^[34-36]. Increasingly, patients with functionally-compensated cirrhosis are being treated, although the likelihood of a good response to treatment is smaller in this group. Since treatment is not contraindicated in any histological group of patients with hepatitis C, the role of such non-invasive technologies should be questioned. It is possible that the evaluation of liver stiffness may provide risk assessment on the basis of which further investigations should be planned. Such a scheme may allow a period of “watchful waiting” prior to a decision on starting treatment, in addition to providing reassurance to patients with mild disease. Another area of interest is the patient’s response to treatment. Preliminary data indicates that LS decreases in patients with hepatitis C after treatment with pegylated interferon and ribavirin and that the decrease is greater in virologic responders compared to non-responders^[37]. This alteration may reflect biochemical changes associated with disease activity, as opposed to changes in fibrosis *per se*, and the long term outcome data in a large cohort of patients is awaited.

Nahon and colleagues addressed the issue of how LSM may affect clinical assessment by inviting four physicians to predict the fibrosis stage on the basis of routine history, physical examination and biochemical tests^[38]. Liver stiffness measurement was performed at the time of liver biopsy. The physicians were allowed to modify their estimate of disease severity in the light of the LSM findings, which were then compared with the biopsy results. LSM did not significantly enhance the physicians’ prediction of pre-cirrhotic disease staging compared to the assessment based on routine investigations. In the

prediction of cirrhosis, the addition of LSM improved the diagnostic accuracy by about 10% in results obtained by 3 out of the 4 physicians.

The utility of TE in routine clinical assessment of precirrhotic disease appears questionable in the light of data currently available. Routine abdominal ultrasound is carried out in nearly all patients with chronic liver disease to assess structural abnormalities, and to look for cholestasis, portal hypertension and hepatocellular carcinoma. The presence of findings such as increased heterogeneity, irregular liver outline or nodularity, caudate lobe hypertrophy, increased spleen size, and portal vein Doppler blood flow measurement all provide evidence of cirrhosis, with high specificity, but relatively low sensitivity^[39,40]. It is possible that routine ultrasound enhances the physician's assessment of whether a patient has cirrhosis, while in addition providing the additional information described above. However, it should be noted that in the study by Nahon and colleagues, of the 15 patients misclassified as cirrhosis after LSM by the senior physicians, 3 had features of cirrhosis on ultrasound or endoscopy^[38]. It is not clear how many of the patients correctly classified on the basis of LSM would have also been correctly classified had routine ultrasound information been provided. Therefore, before LSM is recommended for routine clinical use, the existing technologies should be compared by employing analogous methods of analysis.

CONCLUSION

Assessment of TE using FibroScan is a novel technique that has been evaluated in a number of well-conducted studies. It is a safe, acceptable and quick technique that provides an objective and reproducible measure of liver stiffness. LSM correlates with histological fibrosis score, but the cut-off values vary depending on the study referred to, the aetiology of the disease and the sensitivity and specificity required. LSM provides high diagnostic accuracy for the detection of cirrhosis, making it a potentially useful tool for population-based screening for cirrhosis in areas of high prevalence. However, the delineation of precirrhotic stages is less clear, although LSM compares well with serological markers of fibrosis such as the APRI score and FibroTest^[19]. LSM has a number of drawbacks which include that it is technically challenging in obese individuals, where it is associated with reduced success rate; it is not possible to perform in the presence of significant ascites; and the effect of marked steatosis has not been addressed.

Recent studies have begun to address the likely place of LSM in routine clinical practice and the impact it may have on physicians' assessment of disease severity. However, several important questions remain to be resolved including: what are the relative contributions of fibrosis and haemodynamic alterations on LSM? How does steatosis affect LSM? Which cut-off values should be used for which indication? Does LSM substantially add to the information already obtained by routine clinical assessment, abdominal ultrasound and simple blood tests such as the APRI score? Is LSM sufficiently sensitive to detect changes in fibrosis over the long term; in terms of both disease progression and response to treatment?

While such information is awaited, LSM is being performed increasingly worldwide as a result of increased awareness of the technique. The following recommendations are made for consideration when employing the technique: (1) As different cut-off values exist for different diseases, the diagnosis should always be obtained prior to interpretation of the LSM. (2) The clinical question should be defined in order that a cut-off value is used incorporating the appropriate sensitivity and specificity. (3) Criteria in terms of success rates and minimum number of readings should be defined so that assumptions are not made on the basis of inadequate data. (4) Comparison should be made with liver biopsy, if available, in order to provide continual validation. (5) The process of continuous audit should be instituted, with particular emphasis on how such measurements influence decision-making.

THE FUTURE OF IMAGING BIOMARKERS

Fibrosis and fibrogenesis is a complex multistep process. It would be surprising if a single biomarker was able to provide complete evaluation of the disease. FibroScan is an innovative and user-friendly technology but, despite strong academic and commercial promotion, its limitations have been described in a several well-conducted studies. Assessment of precirrhotic disease and the longitudinal assessment of change in fibrosis have not been fully evaluated. A comprehensive, non-invasive assessment of chronic liver disease will be very helpful for baseline assessment of disease and to evaluate the impact of new antifibrotic therapies. Serum panel markers and imaging techniques including ultrasound and magnetic resonance modalities need to be investigated longitudinally in a number of disease states in order to develop and identify the most effective combination of tests, of which TE with FibroScan may be one. The challenge is to develop and validate such a protocol, and to correlate the results with clinically meaningful outcome measures.

ACKNOWLEDGMENTS

The authors thank Jane Cox, Mary Crossey, Philip Murphy, Nayna Patel, Howard Thomas, Mark Thursz and Caroline Wooldridge for useful discussions.

REFERENCES

- 1 Friedman SL, Bansal MB. Reversal of hepatic fibrosis—fact or fantasy? *Hepatology* 2006; **43**: S82-S88
- 2 Guha IN, Parkes J, Roderick PR, Harris S, Rosenberg WM. Non-invasive markers associated with liver fibrosis in non-alcoholic fatty liver disease. *Gut* 2006; **55**: 1650-1660
- 3 Parkes J, Guha IN, Roderick P, Rosenberg W. Performance of serum marker panels for liver fibrosis in chronic hepatitis C. *J Hepatol* 2006; **44**: 462-474
- 4 Poynard T, Imbert-Bismut F, Munteanu M, Messous D, Myers RP, Thabut D, Ratzu V, Mercadier A, Benhamou Y, Hainque B. Overview of the diagnostic value of biochemical markers of liver fibrosis (FibroTest, HCV FibroSure) and necrosis (ActiTest) in patients with chronic hepatitis C. *Comp Hepatol* 2004; **3**: 8
- 5 Cobbold J, Lim A, Wylezinska M, Cunningham C, Crossey

- M, Thomas H, Patel N, Cox J, Taylor-Robinson S. Magnetic resonance and ultrasound techniques for the evaluation of hepatic fibrosis. *Hepatology* 2006; **43**: 1401-1402; author reply 1402
- 6 **Lim AK**, Taylor-Robinson SD, Patel N, Eckersley RJ, Goldin RD, Hamilton G, Foster GR, Thomas HC, Cosgrove DO, Blomley MJ. Hepatic vein transit times using a microbubble agent can predict disease severity non-invasively in patients with hepatitis C. *Gut* 2005; **54**: 128-133
- 7 **Lim AK**, Patel N, Hamilton G, Hajnal JV, Goldin RD, Taylor-Robinson SD. The relationship of in vivo 31P MR spectroscopy to histology in chronic hepatitis C. *Hepatology* 2003; **37**: 788-794
- 8 **Albrecht T**, Blomley MJ, Cosgrove DO, Taylor-Robinson SD, Jayaram V, Eckersley R, Urbank A, Butler-Barnes J, Patel N. Non-invasive diagnosis of hepatic cirrhosis by transit-time analysis of an ultrasound contrast agent. *Lancet* 1999; **353**: 1579-1583
- 9 **Blomley MJ**, Lim AK, Harvey CJ, Patel N, Eckersley RJ, Basilico R, Heckemann R, Urbank A, Cosgrove DO, Taylor-Robinson SD. Liver microbubble transit time compared with histology and Child-Pugh score in diffuse liver disease: a cross sectional study. *Gut* 2003; **52**: 1188-1193
- 10 **Dezortova M**, Taimr P, Skoch A, Spicak J, Hajek M. Etiology and functional status of liver cirrhosis by 31P MR spectroscopy. *World J Gastroenterol* 2005; **11**: 6926-6931
- 11 **Chappell KE**, Patel N, Gatehouse PD, Main J, Puri BK, Taylor-Robinson SD, Bydder GM. Magnetic resonance imaging of the liver with ultrashort TE (UTE) pulse sequences. *J Magn Reson Imaging* 2003; **18**: 709-713
- 12 **Aubé C**, Racineux PX, Lebilot J, Oberti F, Croquet V, Argaud C, Calès P, Caron C. Diagnosis and quantification of hepatic fibrosis with diffusion weighted MR imaging: preliminary results. *J Radiol* 2004; **85**: 301-306
- 13 **Sandrin L**, Fourquet B, Hasquenoph JM, Yon S, Fournier C, Mal F, Christidis C, Ziol M, Poulet B, Kazemi F, Beaugrand M, Palau R. Transient elastography: a new noninvasive method for assessment of hepatic fibrosis. *Ultrasound Med Biol* 2003; **29**: 1705-1713
- 14 **Intraobserver and interobserver variations in liver biopsy interpretation in patients with chronic hepatitis C**. The French METAVIR Cooperative Study Group. *Hepatology* 1994; **20**: 15-20
- 15 **Ziol M**, Handra-Luca A, Kettaneh A, Christidis C, Mal F, Kazemi F, de Ledinghen V, Marcellin P, Dhumeaux D, Trinchet JC, Beaugrand M. Noninvasive assessment of liver fibrosis by measurement of stiffness in patients with chronic hepatitis C. *Hepatology* 2005; **41**: 48-54
- 16 **Bedossa P**, Dargère D, Paradis V. Sampling variability of liver fibrosis in chronic hepatitis C. *Hepatology* 2003; **38**: 1449-1457
- 17 **Ganne-Carrié N**, Ziol M, de Ledinghen V, Douvin C, Marcellin P, Castera L, Dhumeaux D, Trinchet JC, Beaugrand M. Accuracy of liver stiffness measurement for the diagnosis of cirrhosis in patients with chronic liver diseases. *Hepatology* 2006; **44**: 1511-1517
- 18 **Ishak K**, Baptista A, Bianchi L, Callea F, De Groote J, Gudat F, Denk H, Desmet V, Korb G, MacSween RN. Histological grading and staging of chronic hepatitis. *J Hepatol* 1995; **22**: 696-699
- 19 **Castéra L**, Vergniol J, Foucher J, Le Bail B, Chanteloup E, Haaser M, Darriet M, Couzigou P, De Ledinghen V. Prospective comparison of transient elastography, Fibrotest, APRI, and liver biopsy for the assessment of fibrosis in chronic hepatitis C. *Gastroenterology* 2005; **128**: 343-350
- 20 **Corpechot C**, El Naggar A, Poujol-Robert A, Ziol M, Wendum D, Chazouillères O, de Ledinghen V, Dhumeaux D, Marcellin P, Beaugrand M, Poupon R. Assessment of biliary fibrosis by transient elastography in patients with PBC and PSC. *Hepatology* 2006; **43**: 1118-1124
- 21 **Carrión JA**, Navasa M, Bosch J, Bruguera M, Gilibert R, Forns X. Transient elastography for diagnosis of advanced fibrosis and portal hypertension in patients with hepatitis C recurrence after liver transplantation. *Liver Transpl* 2006; **12**: 1791-1798
- 22 **Gómez-Domínguez E**, Mendoza J, Rubio S, Moreno-Monteagudo JA, García-Buey L, Moreno-Otero R. Transient elastography: a valid alternative to biopsy in patients with chronic liver disease. *Aliment Pharmacol Ther* 2006; **24**: 513-518
- 23 **de Ledinghen V**, Douvin C, Kettaneh A, Ziol M, Roulot D, Marcellin P, Dhumeaux D, Beaugrand M. Diagnosis of hepatic fibrosis and cirrhosis by transient elastography in HIV/hepatitis C virus-coinfected patients. *J Acquir Immune Defic Syndr* 2006; **41**: 175-179
- 24 **Fraquelli M**, Rigamonti C, Casazza G, Conte D, Donato MF, Ronchi G, Colombo M. Reproducibility of transient elastography in the evaluation of liver fibrosis in patients with chronic liver disease. *Gut* 2007; **56**: 968-973
- 25 **Foucher J**, Chanteloup E, Vergniol J, Castéra L, Le Bail B, Adhoute X, Bertet J, Couzigou P, de Ledinghen V. Diagnosis of cirrhosis by transient elastography (FibroScan): a prospective study. *Gut* 2006; **55**: 403-408
- 26 **Kawamoto M**, Mizuguchi T, Katsuramaki T, Nagayama M, Oshima H, Kawasaki H, Nobuoka T, Kimura Y, Hirata K. Assessment of liver fibrosis by a noninvasive method of transient elastography and biochemical markers. *World J Gastroenterol* 2006; **12**: 4325-4330
- 27 **Vizzutti F**, Arena U, Romanelli RG, Rega L, Foschi M, Colagrande S, Petrarca A, Moscarella S, Belli G, Zignego AL, Marra F, Laffi G, Pinzani M. Liver stiffness measurement predicts severe portal hypertension in patients with HCV-related cirrhosis. *Hepatology* 2007; **45**: 1290-1297
- 28 **Coco B**, Oliveri F, Colombatto P, Ciccorossi P, Sacco R, Bonino F, Brunetto MR. Liver Stiffness Measured By Transient Elastography: The Influence of Biochemical Activity. *J Hepatol* 2006; **44** Suppl: S196-S196
- 29 **Lonardo A**, Adinolfi LE, Loria P, Carulli N, Ruggiero G, Day CP. Steatosis and hepatitis C virus: mechanisms and significance for hepatic and extrahepatic disease. *Gastroenterology* 2004; **126**: 586-597
- 30 **Foucher J**, Castéra L, Bernard PH, Adhoute X, Laharie D, Bertet J, Couzigou P, de Ledinghen V. Prevalence and factors associated with failure of liver stiffness measurement using FibroScan in a prospective study of 2114 examinations. *Eur J Gastroenterol Hepatol* 2006; **18**: 411-412
- 31 **Kazemi F**, Kettaneh A, N'kontchou G, Pinto E, Ganne-Carrie N, Trinchet JC, Beaugrand M. Liver stiffness measurement selects patients with cirrhosis at risk of bearing large oesophageal varices. *J Hepatol* 2006; **45**: 230-235
- 32 **Masaki N**, Imamura M, Kikuchi Y, Oka S. Usefulness of elastometry in evaluating the extents of liver fibrosis in hemophiliacs coinfectd with hepatitis C virus and human immunodeficiency virus. *Hepatol Res* 2006; **35**: 135-139
- 33 **Posthouwer D**, Mauser-Bunschoten EP, Fischer K, VAN Erpecum KJ, DE Knecht RJ. Significant liver damage in patients with bleeding disorders and chronic hepatitis C: non-invasive assessment of liver fibrosis using transient elastography. *J Thromb Haemost* 2007; **5**: 25-30
- 34 **Wright M**, Grieve R, Roberts J, Main J, Thomas HC. Health benefits of antiviral therapy for mild chronic hepatitis C: randomised controlled trial and economic evaluation. *Health Technol Assess* 2006; **10**: 1-113, iii
- 35 **Grieve R**, Roberts J, Wright M, Sweeting M, DeAngelis D, Rosenberg W, Bassendine M, Main J, Thomas H. Cost effectiveness of interferon alpha or peginterferon alpha with ribavirin for histologically mild chronic hepatitis C. *Gut* 2006; **55**: 1332-1338
- 36 **National Institute for Clinical Excellence (NICE)**. Pegylated interferon alfa and ribavirin for the treatment of mild hepatitis C. August 2006; Available from: URL: <http://guidance.nice.org.uk/TA106/guidance/word/English>
- 37 **Grando-Lemaire V**, De Ledinghen V, Bourcier V, Ganne-Carrie N, Trinchet JC, Beaugrand M. Liver stiffness measurement (LSM) as a tool to measure liver fibrosis in treated patients with chronic hepatitis C (CHC). *J Hepatol* 2006; **44** Suppl: S214

- 38 **Nahon P**, Thabut G, Ziol M, Htar MT, Cesaro F, Barget N, Grando-Lemaire V, Ganne-Carrie N, Trinchet JC, Beaugrand M. Liver stiffness measurement versus clinicians' prediction or both for the assessment of liver fibrosis in patients with chronic hepatitis C. *Am J Gastroenterol* 2006; **101**: 2744-2751
- 39 **Aubé C**, Winkfield B, Oberti F, Vuillemin E, Rousselet MC, Caron C, Calès P. New Doppler ultrasound signs improve the non-invasive diagnosis of cirrhosis or severe liver fibrosis. *Eur J Gastroenterol Hepatol* 2004; **16**: 743-751
- 40 **Colli A**, Fraquelli M, Andreoletti M, Marino B, Zuccoli E, Conte D. Severe liver fibrosis or cirrhosis: accuracy of US for detection--analysis of 300 cases. *Radiology* 2003; **227**: 89-94

S- Editor Ma N **L- Editor** Anand BS **E- Editor** Yin DH

EDITORIAL

Stimulating erythropoiesis in inflammatory bowel disease associated anemia

Georgia Tsiolakidou, Ioannis E Koutroubakis

Georgia Tsiolakidou, Ioannis E Koutroubakis, Department of Gastroenterology University Hospital Heraklion, Crete, Greece
Correspondence to: Ioannis E Koutroubakis, MD, PhD, Assistant Professor of Medicine, Department of Gastroenterology, University Hospital Heraklion, PO BOX 1352, Heraklion 71110, Crete, Greece. ikoutroub@med.uoc.gr
Telephone: +30-28-10392687 Fax: +30-28-10542085
Received: June 22, 2007 Revised: July 9, 2007

Abstract

Anemia is a frequent complication in patients with inflammatory bowel disease (IBD), and is associated with decreased quality of life and increased rate of hospitalization. The primary therapeutic targets of IBD-associated anemia are iron deficiency and anemia of chronic disease. An important prognostic parameter of the success or failure of therapy is the outcome of the underlying disease. Iron deficiency should be appropriately managed with iron supplementation. However, the use of oral iron therapy is limited by several problems, the most important being gastrointestinal side effects leading occasionally to disease relapse and poor iron absorption. Intravenous iron preparations are more reliable, with iron sucrose demonstrating the best efficacy and tolerability. Treatment with erythropoietin or darbepoetin has been proven to be effective in patients with anemia, who fail to respond to intravenous iron. Patients with ongoing inflammation have anemia of chronic disease and may require combination therapy comprising of intravenous iron sucrose and erythropoietin. After initiating treatment, careful monitoring of hemoglobin levels and iron parameters is needed in order to avoid recurrence of anemia. In conclusion, anemia in the setting of IBD should be aggressively diagnosed, investigated, and treated. Future studies should define the optimal dose and schedule of intravenous iron supplementation and appropriate erythropoietin therapy in these patients.

© 2007 WJG. All rights reserved.

Key words: Anemia; Crohn's disease; Erythropoiesis; Erythropoietin; Iron; Ulcerative colitis

Tsiolakidou G, Koutroubakis IE. Stimulating erythropoiesis in inflammatory bowel disease associated anemia. *World J Gastroenterol* 2007; 13(36): 4798-4806

<http://www.wjgnet.com/1007-9327/13/4798.asp>

INTRODUCTION

Anemia is a frequent and serious complication in patients with inflammatory bowel disease (IBD), with reported prevalence varying from 6% to 74%^[1,2]. Anemia is associated with a decrease in the quality of life and increased rate of hospitalization^[3,4]. In the past, it was common to ascribe anemia as an unavoidable accompaniment of IBD, but recently, correction of anemia is being emphasized as a specific therapeutic objective in these patients^[5,6]. Both iron deficiency and anemia of chronic disease contribute to the development of anemia in IBD^[3]. The therapeutic targets in the treatment of IBD patients with anemia are iron deficiency and the mechanisms underlying anemia of chronic disease^[3].

Anemia is defined as "a reduction in the number of circulating RBCs, hemoglobin concentration, or the volume of packed red cells (hematocrit) in the blood"^[7]. The World Health Organization (WHO) specifies the laboratory definition of anemia as hemoglobin below 120 g/L (non-pregnant females), 110 g/L (pregnant women) and 130 g/L (men). A hemoglobin level below 100 g/L is commonly considered as severe anemia and should be aggressively treated. However, in clinical practice, the timing of therapeutic intervention is based not only on the hemoglobin level but also on the relative degree of hemoglobin reduction, the underlying comorbidities, and the presence or absence of symptoms.

Anemia is an important manifestation of IBD and patients with anemia have increased disease severity and reduced quality of life, indicating the need for effective treatment. The underlying cause of anemia in IBD is either iron deficiency, resulting from intestinal bleeding due to mucosal inflammation and ulceration, or the so-called anemia of chronic disease, resulting from inhibition or suppression of erythropoiesis and dysfunction of iron transport, mediated by inflammatory cytokines^[3,4]. Thus, the pathogenesis of IBD-associated anemia is complex and represents an example of combined iron deficiency anemia (IDA) and anemia of chronic diseases (ACD). Cobalamin or folate deficiency, drug induced anemia, and other causes of anemia such as haemolysis are seen less frequently.

Most patients with IBD-associated anemia respond to iron therapy. Oral iron supplementation appears to be effective for short periods but drug intolerance leads to discontinuation of therapy in up to 21% of patients^[8]. Moreover, the use of oral iron is associated with several limitations^[3]. By contrast, intravenous administration of iron sucrose has been found to be an effective treatment in these patients^[9,10]. Erythropoietin (EPO) administration

is associated with beneficial results in patients who are refractory to oral iron^[3]. The combination of iron sucrose and EPO has been proposed as the most efficacious treatment in IBD-associated anemia^[6,11]. The aim of the present review is to transfer our knowledge, especially the strategies for stimulating erythropoiesis, into the current clinical practice of treatment of anemia in IBD.

ANEMIA IN IBD

Iron deficiency (ID) is the most common cause of anemia in IBD. Iron, which is present in all mammalian cells, is of pivotal importance not only for oxygen transport and storage but also for many non-hematological functions. In normal subjects, the daily iron loss is 1-2 mg which needs to be replaced from the diet. The causes of iron deficiency are reduced intake, either from dietary deficiency or malabsorption, or increased losses. Chronic intestinal bleeding in IBD may exceed the quantity of iron that is absorbed from the diet, resulting in a negative iron balance^[12]. Such an imbalance occurs frequently in IBD, leading to anemia.

When the rate of iron supply to the developing erythroblast is reduced as a result of ID, red cell haemoglobinisation is impaired. The red cells emerging from the bone marrow are microcytic and hypochromic. The increase in EPO response secondary to a drop in haemoglobin levels stimulates erythropoiesis, creating an even greater demand for iron, which is unmet. As a result, there is a high degree of ineffective erythropoiesis^[13]. The most appropriate definition of iron deficiency is bone marrow proliferation in response to intravenous iron supplementation, an approach that has not been studied in IBD. Moreover, in addition to a possible iron deficiency in anemia of chronic disease, functional iron deficiency occurs because of intense erythropoiesis^[14,15] during therapy with erythropoietic agents, with a decrease in transferrin saturation and serum ferritin. Furthermore, during erythropoietin therapy, the absorption of iron increases by as much as 5 fold^[16].

After iron deficiency, anemia of chronic disease is the next important cause of anemia in these patients. There is general correlation between disease activity and the severity of anemia. Abnormal iron metabolism and improper use of iron stores are the typical features of anemia of chronic disease, characterized by low circulating iron concentration in the presence of ample reticuloendothelial iron stores. Thus, anemia of chronic disease can be easily diagnosed by the presence of hypoferraemia and increased serum levels of ferritin.

Because of differences in the therapeutic response, it is important to distinguish anemia of chronic disease from iron deficiency. Iron parameters need to be checked regularly. When both ID and ACD are present, many of the laboratory measures of iron status become unreliable. Circulating iron concentrations are subnormal in both situations. Measurement of serum ferritin reflects the level of iron in the body stores. When serum ferritin is less than 15 µg/L, iron stores are definitely depleted. When ferritin is less than 30 µg/L there is a strong suggestion of iron deficiency, whereas, a value > 200 µg/L indicates

that iron deficiency is unlikely. Moreover, diagnosis of iron deficiency in the setting of IBD is difficult and no single laboratory test is reliable. Undoubtedly, low serum ferritin levels are indicative of iron deficiency. However, in the presence of inflammation, ferritin levels may increase despite iron deficiency. Furthermore, in IBD patients the diagnostic criteria for iron deficiency depend on the level of inflammation, thus in quiescent disease a serum ferritin < 30 µg/L or TfS < 15% indicates iron deficiency, whereas, in active disease a value < 100 µg/L suggests iron deficiency. A combination of true iron deficiency and anemia of chronic disease is possible when the ferritin level is between 30 and 100 g/L. If necessary, a bone marrow aspirate can be examined, which will show absence of macrophage iron in iron-deficient subjects. The ratio of soluble transferrin receptor/log ferritin has also been proposed as a marker for differentiating ID from ACD^[17]. A ratio above 2 is suggestive of iron deficiency, whereas a ratio of less than 1 indicates anemia of chronic disease.

IRON TREATMENT

Since iron deficiency is the most prevalent cause of IBD-associated anemia, iron supplementation is the most relevant therapeutic intervention.

Oral iron preparations

Treatment of anemia in IBD with oral iron is limited by poor absorption, drug intolerance, and induction of oxidative stress at the site of bowel inflammation^[3]. Moreover, there is evidence that oral iron may increase the disease activity in IBD. Oral iron supplements commonly contain iron in the form of ferrous salts (ferrous sulphate, ferrous gluconate, and ferrous fumarate). All ferrous compounds are oxidized in the gut lumen or within the mucosa with the release of activated hydroxyl radicals, which act on the gut wall and produce a range of gastrointestinal symptoms such as nausea, bloating, diarrhea and upper abdominal pain. Absorption of iron appears to be reduced in IBD. The acute phase protein hepcidin may play a central role in this process as it is over expressed in the liver leading to reduced iron uptake by the duodenum^[18]. Moreover, oral iron enhances intestinal inflammation as well as colon carcinogenesis in animal models of colitis^[19,20].

Intravenous iron preparations

Patients with IBD and anemia respond well to intravenous iron therapy with an increase in hemoglobin levels^[9]. Administration of iron directly into the circulation requires formulations that prevent cellular toxicity of iron salts^[21]. Three different products are available: Iron dextran, iron gluconate and iron sucrose. The stability of the dextran complex allows administration of large doses at a single setting. The molecule however may cause dextran-induced anaphylactic reactions. On the other hand, iron gluconate may cause the so-called transient capillary leak syndrome. Non-transferrin bound free ionic iron may induce acute endothelial cell injury, with the development of symptoms such as nausea, hypotension, tachycardia, dyspnoea, and oedema of the hands and feet. Iron sucrose is safer

than iron dextran and is well tolerated even by patients who have previously reacted adversely to iron dextran^[22]. Single doses of up to 300 mg of iron sucrose are safe^[23]. The maximal recommended dose is 600 mg/wk^[24]. If the infusion speed is too rapid, or the single total iron dose is too high, non-transferrin bound free iron may cause transient hypotension, tachycardia and dyspnoea, as described with iron gluconate.

Recently, a new intravenous iron preparation, ferric carboxymaltose (FeCarb) has been introduced. FeCarb can be administered intravenously in single or multiple doses of up to 1000 mg within 15 min. A recent report suggests that administration of FeCarb in IBD-associated anemia provides faster hemoglobin response, a greater increase in iron stores and better patient tolerance^[25]. Intravenous iron therapy for IBD-associated anemia has been recommended since the 1970s^[26], but clinical trials were only performed in the early 1990s^[6]. During the last several years, experience in the use of intravenous iron sucrose in IBD has evolved greatly. Gasche *et al* demonstrated the efficacy of such form of iron supplementation^[6,27,28]. Moreover, intravenous iron sucrose has a good safety profile in IBD-associated anemia, with a 65%-75% response rate in 4-8 wk, associated with improvement in the quality of life^[6]. In IBD patients, high levels of serum transferrin, soluble transferrin receptor, and serum EPO predict the response to intravenous iron supplementation, while low levels indicate the need for concomitant EPO therapy^[9]. Two comparative trials (oral *vs* intravenous iron) have recently been published^[29,30], indicating better tolerability of iv iron sucrose therapy.

The current management of IBD-associated anemia with iron sucrose is based on infusing the total iron dose by multiple small infusions, since a single dose should not exceed 500 mg (or 7 mg/kg body weight). Depending on the body weight, the usual dose for single infusions varies from 200 mg to 300 mg of iron sucrose in 100 mL sodium chloride. The total iron infusion may take between 4 to 10 wk. After this period, about 25% patients will fail to show a significant hemoglobin increase, defined as an increase in hemoglobin concentration of ≥ 20 g/L at the end of the wk 4.

One limitation of intravenous iron in patients not receiving EPO therapy is that much of the administered iron is transported into the reticuloendothelial system as storage iron, where it is less readily available for erythropoiesis.

Safety issues

Intravenous iron therapy has been carefully analyzed for risks and adverse events. Iron is an essential nutrient for proliferating microorganisms, and the sequestration of iron from microorganisms into the reticuloendothelial system is a potentially effective defense strategy against pathogens^[31]. In addition, iron therapy in the setting of long-term immune activation promotes the formation of highly toxic hydroxyl radicals that can cause tissue damage and endothelial dysfunction, with increased risk of acute cardiovascular events^[31-33]. Thus, intravenous iron therapy may predispose patients to infections and unfavorable

coronary outcomes.

Iron therapy is currently not recommended for patients with anemia of chronic disease who have high or normal ferritin levels (above 100 g/L), because of the risk of possible adverse outcome.

ERYTHROPOIETIN AND ANEMIA IN IBD

Erythropoietin, a glycoprotein hormone secreted by the kidney, is the primary growth factor regulating erythropoiesis^[34]. Erythropoiesis must maintain a steady state level of circulating RBCs and in addition respond to acute challenges. The bone marrow is a highly dynamic organ that produces two to three million red cells every second. These red cells contain hemoglobin and are replaced after 75-150 d^[3]. Any imbalance in the rates of red-cell loss and production due to inadequate release of EPO, absence of co-factors required for red-cell formation (in particular iron), or an impaired ability of the erythroid progenitor cells to respond to EPO, results in anemia^[35]. Erythropoiesis is controlled by the hypoxia sensing mechanism in the kidney which responds by modulating the output of EPO^[36].

EPO acts on committed erythroid progenitor cells in the bone marrow to regulate their proliferation, to promote their differentiation and to maintain their viability as they differentiate. Thus, EPO is the major regulator of erythropoiesis^[37]. Under normal physiological conditions, EPO expression is inversely related to tissue oxygenation and hemoglobin levels, and there is a semilogarithmic relationship between the EPO response (log) and the degree of anemia (linear)^[38]. Moreover, the concentration of EPO that is normally between 5 to 29 U/L, can increase 100-fold in the presence of severe anemia. However, in some patients, EPO concentrations fail to increase despite significant anemia^[39].

Measurement of serum EPO levels is useful only in anemic patients with hemoglobin levels less than 100 g/L, since EPO levels at higher hemoglobin concentrations remain well within the normal range^[40]. Serum EPO levels that are inappropriately low for the degree of anemia, indicates blunted EPO response, and is encountered in anemia of chronic disease^[41].

Higher serum EPO levels have been reported in IBD patients compared to the normal population^[39,42,43]. The EPO levels increase with the degree of anemia^[27]. Interestingly, it has been shown that in IBD-associated anemia, EPO production is inadequate in relation to the degree of anemia^[5,9]. This finding may be of help when considering EPO therapy^[9].

Although anemia is a frequent complication of many diseases, its clinical relevance and the importance of its correction have long been neglected. Anemia was found to have a marked effect not only on the quality of life, but also on various physiological functions^[44-46]. Before the mid-1980s there was no effective therapeutic means of stimulating erythropoiesis. The clinical use of EPO began in 1986 with treatment of patients with chronic kidney disease^[47,48]. Since then, indications for the use of EPO therapy to boost erythropoiesis have broadened considerably.

ERYTHROPOIETIC AGENTS

Three erythropoietic agents are currently available in routine clinical practice: epoetin alfa, epoetin beta, and darbepoetin alfa, which differ in terms of their pharmacologic modifications, receptor-binding affinity, and serum half-life, thus allowing for alternative dosing and scheduling strategies^[49].

Recombinant Human Erythropoietin

Epoetin- α and Epoetin- β are the two available brands of recombinant human erythropoietin (rHu EPO). In 1977, small amounts of human EPO were obtained from the urine of patients with aplastic anemia^[50]. Based on limited peptide sequence information of this purified material, the gene of human EPO was isolated and cloned in 1983^[51], and the use of genetic engineering techniques allowed large-scale production of recombinant human EPO in a suitable mammalian cell line. The clinical success of this product has resulted in the use of epoetin in millions of anemic patients.

Like the endogenous hormone, epoetin binds to the dimerised EPO receptor on the surface of erythroid progenitor cells, inducing a conformational change in the receptor. This change induces phosphorylation of tyrosine residues by JAK-2 kinase on several intracellular molecules, including STAT-5, which is the major signal transducer and activator of transcription, causing gene activation in the cell nucleus^[52]. Activation of these pathways stimulates proliferation and inhibits apoptosis of the erythroid progenitor cells^[53].

Epoetin is highly effective in stimulating erythropoiesis^[5,6,54,55]. In addition there is increasing experimental evidence that EPO has a range of non-erythropoietic, pleiotropic effects, including tissue protection of the nervous system, myocardium, kidneys, intestines and the joints^[56].

Darbepoetin alfa

Despite the undoubted therapeutic efficacy of epoetin, a major limitation of this treatment is that it has to be administered parenterally two or three times a week. Much effort has therefore been directed at producing longer-acting EPO analogues that would retain their biological activity, but require less frequent dosing. The first to be synthesized was darbepoetin (DPO) alfa^[57]. DPO alfa has five N-linked glycosylation chains compared with the endogenous and recombinant EPO, both of which have three. All three molecules have, additionally, a single O-linked glycosylation chain^[58]. The molecular weight of DPO alfa is 37.1 kDa, compared with 30.4 kDa for EPO, and its elimination half-life in humans after an intravenous injection is about three-fold longer (25.3 h *vs* 8.5 h for epoetin alfa)^[59]. This allows less frequent dosing, with most patients receiving injections once weekly or once every 2-3-4 wk^[60].

Continuous erythropoietin receptor activator

Continuous EPO receptor activator (CERA) is the another erythropoietic agent, that has recently completed phase III of its clinical development program^[61,62]. CERA was developed by the integration of a single 30 kDa

polymer chain into the EPO molecule, thus, increasing the molecular weight to twice that of epoetin to about 60 kDa, and considerably increasing the elimination half-life in humans to about 130 h. The hypothesis being tested in phase III clinical studies is whether CERA can be administered safely and effectively every 3-4 wk. The preliminary data suggests that this is perhaps the case^[63,64].

Next generation erythropoietic analogues

Synthetic erythropoiesis protein, EPO fusion protein and EPO-mimetic peptides are the next generation of erythropoietic agents that exploit recent advances in drug development for stimulating erythropoiesis. These agents stimulate erythropoiesis through the activation of EPO receptors^[65-67].

ROLE OF ERYTHROPOIETIN IN IBD-ASSOCIATED ANEMIA

Hemoglobin increase

Several studies have examined the efficacy and the safety of EPO in the treatment of refractory anemia in patients with IBD^[5,6,11,68,69]. Horina *et al* first tested tEPO therapy in three patients with a long-standing history of IBD and refractory chronic anemia (Hb < 100 gr/L, plasma EPO < 100 U/L). A marked increase in hemoglobin values was noted in all three patients^[68]. Gasche *et al*^[27] reported that after 5 wk of treatment with intravenous iron alone or in combination with EPO, all anemic patients had a marked increase in hemoglobin levels. However, the mean increase in EPO-treated patients was higher compared to patients receiving iron therapy alone (50 g/L *vs* 20 gr/L respectively). Subsequently, recombinant human EPO was found to be effective in a controlled trial of patients with anemia refractory to oral iron supplementation^[5]. In this double-blind study, the superiority of combination therapy (iron + rHEPO) over iron alone was again demonstrated. In another placebo-controlled study in a patient with Crohn's disease-associated anemia that was refractory to oral iron, intravenous iron, and EPO resulted in greater and quicker hemoglobin response compared with intravenous iron alone^[6]. Concomitant EPO therapy was associated with a more rapid incline in hemoglobin levels. EPO treatment has also been shown to be safe and effective in children with iron refractory IBD-associated anemia^[11].

In a recent study^[70], Darbepoetin-alpha (DPO) was tested in patients with IBD-associated refractory anemia. This study, the first to assess DPO alfa in IBD-associated refractory anemia, demonstrated that the administration of DPO in combination with intravenous iron sucrose raised hemoglobin levels. Furthermore, the longer half-life of DPO compared to EPO allowed less frequent administration and was more convenient to the patients. Thus, all the trials have demonstrated a significant beneficial effect of erythropoietin agents in this patient population.

Improvement in quality of life

Most studies have shown that successful treatment of anemia with EPO, is accompanied with improvement in

the energy and activity level and the overall quality of life^[71]. Surprisingly, in the hemoglobin range of 80-140 g/L, the largest improvement in quality of life occurred when hemoglobin levels increased from 110 to 130 g/L^[72]. Alterations in the quality of life were also examined in anemic patients with Crohn's disease treated with iron sucrose and EPO^[6]. The sense of well being, and improvement in mood, physical ability, and social activity accounted for most of the improvement in quality of life. Moreover, avoidance of blood transfusions enhanced the improvement in quality of life^[15]. Thus, the quality of life is significantly improved in patients with IBD-associated anemia with erythropoietin therapy.

MECHANISMS OF ACTION

The therapeutic effect of EPO involves counteracting the antiproliferative effect of cytokines, along with stimulation of iron uptake and heme biosynthesis in erythroid progenitor cells. Accordingly, a poor response to treatment with erythropoietic agents is associated with increased levels of proinflammatory cytokines, and poor iron availability.

There is little data on the possible effects of therapy with erythropoietic agents and the correction of anemia, on the course of the underlying disease, particularly since epoetin can exert additional biologic effects, including interference with the signal transduction cascade of cytokines. Treatment of rats with EPO was found to reduce the degree of colitis caused by DNBS suggesting that EPO may be useful in IBD treatment^[73]. Furthermore, EPO may enhance the healing of colonic anastomosis after colonic surgery by increasing the number of fibroblasts and accelerating the maturation of new blood vessels^[74].

PREDICTIVE FACTORS OF RESPONSE TO ERYTHROPOIETIC THERAPY

The response (erythropoiesis) to a dose of EPO is not related to the patient's gender or age, suggesting that patient-specific factors such the underlying chronic disease, iron-restricted erythropoiesis, and other factors that normally result in a wide distribution of hemoglobin responses, account for the variability in erythropoietin response to EPO in different individuals^[15].

There are no baseline indices of response to erythropoietin that can be used in routine clinical practice. If functional iron deficiency and vitamin deficiency are excluded, a low serum EPO level appears to be the only established predictive factor of some importance^[75]. In IBD patients, high levels of serum transferrin, soluble transferrin receptor, and serum EPO predict the response to intravenous iron supplementation, while low levels indicate a need for concomitant EPO therapy^[9].

Further studies are needed to investigate the value of hepcidin, C-reactive protein and other measures as predictive factors. The iron-regulatory hormone hepcidin is of particular interest as it is believed to be the primary factor in anemia of chronic disease. Cytokine-mediated induction of hepcidin in inflammatory or infectious

conditions^[76] decreases duodenal absorption of iron and induces iron retention by macrophages. Likewise, CRP is also increased in inflammatory conditions, and high CRP levels are associated with reduced Hb levels and resistance to EPO treatment. Therefore, pre-treatment levels of hepcidin and CRP may provide important information on the response to erythropoietic proteins in IBD patients.

INDICATIONS FOR ERYTHROPOIETIC THERAPY IN IBD-ASSOCIATED ANEMIA

It is difficult to determine as to which patient with IBD-associated refractory anemia will require combination therapy with erythropoietic agents in view of the absence of any long-term data. However, EPO should be reserved for symptomatic patients who may otherwise require blood transfusions, who have not responded to intravenous iron, and in whom aggressive management of IBD (including immunosuppressive therapy) has not suppressed the mucosal inflammation. EPO is an adjunct and not an alternative to appropriate treatment of IBD^[77]. Several issues remain to be resolved including the use of EPO for prevention of anemia, the target hemoglobin levels, and the immunologic role of EPO in the setting of chronic bowel inflammation.

SIDE EFFECTS AND CONCERNS ABOUT EPO THERAPY

Erythropoietic therapy is highly effective in stimulating erythropoiesis and has an excellent safety profile. Apart from the rare induction of antibodies, all adverse effects of epoetin appear to be directly related to its pharmacodynamic properties i.e., the increase in red-cell number. In patients with kidney disease, such an increase may lead to a moderate rise in the blood pressure. In addition, there is a risk of thromboembolic complications with higher hemoglobin levels. Clinical trials in patients with cancer^[78] and chronic kidney disease^[79], designed to assess the effect of increasing the haemoglobin concentration to the normal range, compared with a subnormal target, have suggested no overall benefit and even the risk of potential harm. Moreover, it has recently been observed that "on the basis of available data, the maintenance of haemoglobin concentrations above 130 g/L appears to be unsafe in patients with chronic renal failure"^[80].

The success of epoetin has been clouded by the discovery in a few patients of the development of neutralizing antibodies against recombinant proteins that cross-react with native EPO, and cause pure red-cell aplasia^[81]. This problem is likely caused by the use of a new buffer (polysorbate 80), which replaced human serum albumin, and which induces the release of organic compounds with adjuvant properties from the rubber stoppers of prefilled syringes^[82].

Another issue of concern is the presence of EPO receptors on several malignant cell lines, including mammary, ovarian, uterine, prostate, hepatocellular, and renal carcinomas, as well as on the myeloid cell lines^[83]. There are contradictory reports concerning the effect of

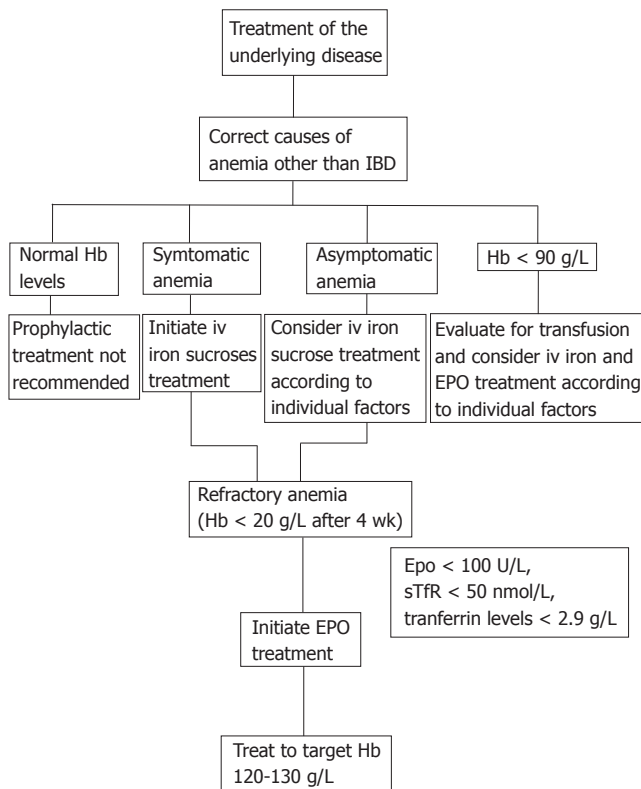


Figure 1 IBD-associated anemia. Suggested treatment algorithm for stimulating erythropoiesis in patients with IBD-associated anemia. Hb: Hemoglobin; iv: Intravenous; EPO: Erythropoietin.

epoetin on such cells. Although the use of EPO caused tumor regression in a murine model of myeloma^[84], administration to EPO-receptor-expressing human renal carcinoma cells *in vitro* stimulated their proliferation^[85]. Large amounts of EPO receptors were found in 90% of biopsy specimens of human breast carcinoma^[86]. The production of EPO receptors by cancer cells appears to be regulated by hypoxia, and in clinical cancer specimens the highest levels of EPO receptors were associated with neoangiogenesis, tumor hypoxia, and presence of infiltrating tumors. One potential adverse effect may be the induction of neoangiogenesis, since EPO increases inflammation and ischemia-induced neovascularization by enhancing the mobilization of endothelial progenitor cells^[87]. In a study in nude mice, implantation of EPO-receptor expressing cell lines and subsequent inhibition of EPO-receptor signaling resulted in inhibition of angiogenesis and destruction of the tumor mass^[83].

The effects of erythropoiesis-stimulating agents are still not fully understood. Not only do they increase haemoglobin concentration, but they may also act *via* alternative dose-dependent pathways that are harmful. Careful studies on the potential harmful effects of EPO therapy in patients with different forms of anemia of chronic disease remain to be carried out.

STRATEGIES FOR STIMULATING ERYTHROPOIESIS IN IBD

Anemia is a common complication of IBD and poses a

serious therapeutic challenge. The optimal management of IBD-associated anemia is to promote an increase in hemoglobin levels accompanied with an improvement in the quality of life. Therapy is targeted at the mechanisms underlying anemia of chronic disease and iron deficiency. The most important approach is to treat the underlying disease (Figure 1). In previous studies assessing EPO in anemic IBD patients, current or recent immunosuppressive therapy was a specific exclusion criterion^[5,6], suggesting that the patients may not be receiving optimal anti-inflammatory therapy. Moreover, as the IBD disease activity correlates with the degree of anemia, treatment of IBD should lower its incidence. Thus, as effective therapy (including immunosuppressants and anti TNF- α) induces mucosal healing^[88,89], one would expect a lower incidence of anemia.

Additionally, causes of anemia other than IBD (nutritional defects, bleeding, haemolysis, chronic kidney disease, cancer, metabolic disorders and cardiovascular disease) should be evaluated and treated. Asymptomatic anemia warrants correction, especially in patients older than 65 years of age, those with additional risk factors (such as coronary artery disease, pulmonary disease, and chronic kidney disease), or a combination of these factors.

A stepwise approach for stimulating erythropoiesis should be followed in patients with IBD. First, iron deficiency which is the most common cause of anemia should be corrected. Recent studies have demonstrated the efficacy and the safety of intravenous iron supplementation with iron sucrose. About two-thirds of patients respond to this treatment within 4-10 wk. The remaining subjects who fail to improve likely have anemia of chronic disease. Gasche *et al* suggest that serum levels of EPO, soluble transferring receptor, and transferrin predict a positive response to iron infusion, and may identify those patients who will benefit from early treatment with EPO^[9].

EPO administration has been shown to be of benefit in patients who do not respond to iron supplementation. Several studies have demonstrated the efficacy and the safety of EPO in IBD patients with refractory anemia. EPO treatment results in a marked increase in hemoglobin values and improvement in quality of life. However, there is controversy as to whether EPO is cost-effective in treating anemia in IBD. Many physicians have employed erythropoietic agents which are not currently approved by the FDA. Thus, clinicians will have to make treatment decisions based on limited data currently available. These agents are effective in increasing hemoglobin level, and improving the quality of life for the duration they are used. However, the length of treatment, optimal dose and target hemoglobin level remain to be established. The disadvantage of these agents is that they add another drug to the patient's treatment regimen, thereby increasing the costs, inconvenience, and potential side effects. Several studies in patients with renal failure and cancer indicate that the target hemoglobin level with EPO should be 120-130 g/L. However, a normal haematocrit value may not be optimal.

IBD-associated anemia can also be managed with blood transfusions which are widely used for rapid and effective intervention. Transfusions are particularly helpful in the context of severe anemia (Hb < 90 g/L)

and life-threatening anemia (Hb < 70 g/L). However, the optimal hemoglobin threshold for red-cell transfusion in IBD patients is unknown. Recent evidence^[90,91] does not support the unrestricted use of blood transfusions, because of the risks associated with this procedure such as iron overload and sensitization of the immune system. Thus, the timing of blood transfusion in IBD-associated anemia must take into consideration not only the hemoglobin level but also the relative degree of hemoglobin decrease, any underlying comorbidities and the presence of anemia-related symptoms. It is also important that the use of blood transfusions should be followed by treatment with iron supplementation, with or without EPO.

In our clinical practice, most patients with IBD-associated anemia respond well to intravenous iron alone. It is important that the bowel inflammation is treated adequately and that sufficient iron is given. In anemic patients with active IBD, we usually start treatment with corticosteroids, in order to alleviate the clinical symptoms, followed by intravenous iron sucrose to replenish the iron stores. This strategy helps to stimulate impaired erythropoiesis and thus reduce the need for concomitant EPO injections. However, patients with ongoing inflammation have anemia of chronic disease and may require combination therapy with intravenous iron sucrose and erythropoietic agents. It has been suggested that about 25% of patients with IBD-associated anemia require combined treatment with iron sucrose and EPO^[8]. Recently, we examined the use of DPO to determine whether it is effective in refractory anemia in IBD^[70]. The administration of DPO in combination with intravenous iron sucrose was effective in these patients. Careful monitoring of hemoglobin levels and iron parameters is required to avoid recurrence of anemia in IBD patients. Additional clinical trials are warranted to establish the optimal dose and schedule of intravenous iron supplementation, and erythropoietic therapy in IBD-associated anemia.

CONCLUSION

Anemia is a frequent complication of IBD. Patients with anemia usually have greater disease severity and lower quality of life, demanding aggressive diagnosis and treatment. Iron deficiency is managed most reliably by intravenous preparations. Iron sucrose demonstrates the best efficacy and tolerability. Difficulties arise in patients with refractory anemia, who do not respond to intravenous iron. EPO administration has proven to be effective in these patients. EPO has additional beneficial effects on hemoglobin concentration. However, EPO is expensive and not without potential side effects. It is difficult to determine as to which patient with refractory anemia will require combination therapy with erythropoietic agents. The responsible use of medical resources, as well as the absence of data on long-term safety of EPO in patients with IBD, suggests that iron sucrose should be considered as the first-line therapy in IBD-associated anemia. Therefore, EPO has a secondary role, in patients who do not respond to intravenous iron alone. The long-term outcome of alleviating anemia depends on whether the bowel inflammation can be adequately treated.

REFERENCES

- 1 Ebinger M, Leidl R, Thomas S, Von Tirpitz C, Reinshagen M, Adler G, König HH. Cost of outpatient care in patients with inflammatory bowel disease in a German University Hospital. *J Gastroenterol Hepatol* 2004; **19**: 192-199
- 2 Werlin SL, Grand RJ. Severe colitis in children and adolescents: diagnosis. Course, and treatment. *Gastroenterology* 1977; **73**: 828-832
- 3 Gasche C, Lomer MC, Cavill I, Weiss G. Iron, anaemia, and inflammatory bowel diseases. *Gut* 2004; **53**: 1190-1197
- 4 Wilson A, Reyes E, Ofman J. Prevalence and outcomes of anemia in inflammatory bowel disease: a systematic review of the literature. *Am J Med* 2004; **116** Suppl 7A: 44S-49S
- 5 Schreiber S, Howaldt S, Schnoor M, Nikolaus S, Bauditz J, Gasché C, Lochs H, Raedler A. Recombinant erythropoietin for the treatment of anemia in inflammatory bowel disease. *N Engl J Med* 1996; **334**: 619-623
- 6 Gasché C, Dejaco C, Waldhoer T, Tillinger W, Reinisch W, Fueger GF, Gangl A, Lochs H. Intravenous iron and erythropoietin for anemia associated with Crohn disease. A randomized, controlled trial. *Ann Intern Med* 1997; **126**: 782-787
- 7 Anemia: A hidden epidemic. Available from: URL: <http://www.anemia.org/professional/monograph>. Accessed June 12 2006
- 8 Kulnigg S, Gasche C. Systematic review: managing anaemia in Crohn's disease. *Aliment Pharmacol Ther* 2006; **24**: 1507-1523
- 9 Gasche C, Waldhoer T, Feichtenschlager T, Male C, Mayer A, Mittermaier C, Petritsch W. Prediction of response to iron sucrose in inflammatory bowel disease-associated anemia. *Am J Gastroenterol* 2001; **96**: 2382-2387
- 10 Bodemar G, Kechagias S, Almer S, Danielson BG. Treatment of anaemia in inflammatory bowel disease with iron sucrose. *Scand J Gastroenterol* 2004; **39**: 454-458
- 11 Dohil R, Hassall E, Wadsworth LD, Israel DM. Recombinant human erythropoietin for treatment of anemia of chronic disease in children with Crohn's disease. *J Pediatr* 1998; **132**: 155-159
- 12 Child JA, Brozović B, Dyer NH, Mollin DL, Dawson AM. The diagnosis of iron deficiency in patients with Crohn's disease. *Gut* 1973; **14**: 642-648
- 13 Cavill I. Erythropoiesis and iron. *Best Pract Res Clin Haematol* 2002; **15**: 399-409
- 14 Brugnara C. Iron deficiency and erythropoiesis: new diagnostic approaches. *Clin Chem* 2003; **49**: 1573-1578
- 15 Goodnough LT, Skikne B, Brugnara C. Erythropoietin, iron, and erythropoiesis. *Blood* 2000; **96**: 823-833
- 16 Skikne BS, Cook JD. Effect of enhanced erythropoiesis on iron absorption. *J Lab Clin Med* 1992; **120**: 746-751
- 17 Punnonen K, Irjala K, Rajamäki A. Serum transferrin receptor and its ratio to serum ferritin in the diagnosis of iron deficiency. *Blood* 1997; **89**: 1052-1057
- 18 Ganz T. Hepcidin, a key regulator of iron metabolism and mediator of anemia of inflammation. *Blood* 2003; **102**: 783-788
- 19 Carrier JC, Aghdassi E, Jeejeebhoy K, Allard JP. Exacerbation of dextran sulfate sodium-induced colitis by dietary iron supplementation: role of NF-kappaB. *Int J Colorectal Dis* 2006; **21**: 381-387
- 20 Seril DN, Liao J, Ho KL, Warsi A, Yang CS, Yang GY. Dietary iron supplementation enhances DSS-induced colitis and associated colorectal carcinoma development in mice. *Dig Dis Sci* 2002; **47**: 1266-1278
- 21 Geisser P, Baer M, Schaub E. Structure/histotoxicity relationship of parenteral iron preparations. *Arzneimittelforschung* 1992; **42**: 1439-1452
- 22 Van Wyck DB, Cavallo G, Spinowitz BS, Adhikarla R, Gagnon S, Charytan C, Levin N. Safety and efficacy of iron sucrose in patients sensitive to iron dextran: North American clinical trial. *Am J Kidney Dis* 2000; **36**: 88-97
- 23 Yee J, Besarab A. Iron sucrose: the oldest iron therapy becomes new. *Am J Kidney Dis* 2002; **40**: 1111-1121
- 24 Chandler G, Harchawal J, Macdougall IC. Intravenous iron sucrose: establishing a safe dose. *Am J Kidney Dis* 2001; **38**: 988-991
- 25 Kulnigg S, Rumyantsev V, Stoinov S, Kulnigg S, Rumyantsev V, Stoinov S, Simanenkova V, Levchenko E,

- Karnafel W, Garcia LC, D'Haens G, Gasche C. A novel intravenous iron formulation for treatment of anemia in IBD: The Ferinject randomized, controlled trial. *Gastroenterology* 2007; **132** suppl 2: S501-S502
- 26 **Bartels U**, Pedersen NS, Jarnum S. Iron absorption and serum ferritin in chronic inflammatory bowel disease. *Scand J Gastroenterol* 1978; **13**: 649-656
 - 27 **Gasché C**, Reinisch W, Lochs H, Parsaei B, Bakos S, Wyatt J, Fueger GF, Gangl A. Anemia in Crohn's disease. Importance of inadequate erythropoietin production and iron deficiency. *Dig Dis Sci* 1994; **39**: 1930-1934
 - 28 **Gasche C**, Dejaco C, Reinisch W, Tillinger W, Waldhoer T, Fueger GF, Lochs H, Gangl A. Sequential treatment of anemia in ulcerative colitis with intravenous iron and erythropoietin. *Digestion* 1999; **60**: 262-267
 - 29 **Schröder O**, Mickisch O, Seidler U, de Weerth A, Dignass AU, Herfarth H, Reinshagen M, Schreiber S, Junge U, Schrott M, Stein J. Intravenous iron sucrose versus oral iron supplementation for the treatment of iron deficiency anemia in patients with inflammatory bowel disease-a randomized, controlled, open-label, multicenter study. *Am J Gastroenterol* 2005; **100**: 2503-2509
 - 30 **Erichsen K**, Ulvik RJ, Nysaeter G, Johansen J, Ostborg J, Berstad A, Berge RK, Hausken T. Oral ferrous fumarate or intravenous iron sucrose for patients with inflammatory bowel disease. *Scand J Gastroenterol* 2005; **40**: 1058-1065
 - 31 **Weinberg ED**. Iron loading and disease surveillance. *Emerg Infect Dis* 1999; **5**: 346-352
 - 32 **Kletzmayer J**, Sunder-Plassmann G, Hörl WH. High dose intravenous iron: a note of caution. *Nephrol Dial Transplant* 2002; **17**: 962-965
 - 33 **Sullivan JL**. Iron therapy and cardiovascular disease. *Kidney Int Suppl* 1999; **69**: S135-S137
 - 34 **Eschbach JW**, Adamson JW. Guidelines for recombinant human erythropoietin therapy. *Am J Kidney Dis* 1989; **14**: 2-8
 - 35 **Macdougall IC**, Eckardt KU. Novel strategies for stimulating erythropoiesis and potential new treatments for anaemia. *Lancet* 2006; **368**: 947-953
 - 36 **Tan CC**, Eckardt KU, Firth JD, Ratcliffe PJ. Feedback modulation of renal and hepatic erythropoietin mRNA in response to graded anemia and hypoxia. *Am J Physiol* 1992; **263**: F474-F481
 - 37 **Jelkmann W**. Erythropoietin: structure, control of production, and function. *Physiol Rev* 1992; **72**: 449-489
 - 38 **Weiss G**, Goodnough LT. Anemia of chronic disease. *N Engl J Med* 2005; **352**: 1011-1023
 - 39 **Tsitsika A**, Stamoulakatou A, Kafritsa Y, Paleologos G, Panayotou I, Premetis E, Roma E, Papassotiropoulos I. Erythropoietin levels in children and adolescents with inflammatory bowel disease. *J Pediatr Hematol Oncol* 2005; **27**: 93-96
 - 40 **Miller CB**, Jones RJ, Piantadosi S, Abeloff MD, Spivak JL. Decreased erythropoietin response in patients with the anemia of cancer. *N Engl J Med* 1990; **322**: 1689-1692
 - 41 **Barosi G**. Inadequate erythropoietin response to anemia: definition and clinical relevance. *Ann Hematol* 1994; **68**: 215-223
 - 42 **Demirtürk L**, Hülagu S, Yaylaci M, Altin M, Ozel M. Serum erythropoietin levels in patients with severe anemia secondary to inflammatory bowel disease and the use of recombinant human erythropoietin in patients with anemia refractory to treatment. *Dis Colon Rectum* 1995; **38**: 896-897
 - 43 **Kapsoritakis AN**, Koukourakis MI, Sfiridaki A, Potamianos SP, Kosmadaki MG, Koutroubaki IE, Kouroumalis EA. Mean platelet volume: a useful marker of inflammatory bowel disease activity. *Am J Gastroenterol* 2001; **96**: 776-781
 - 44 **Macdougall IC**. Quality of life and anemia: the nephrology experience. *Semin Oncol* 1998; **25**: 39-42
 - 45 **Daneryd P**, Svanberg E, Körner U, Lindholm E, Sandström R, Brevinge H, Pettersson C, Bosaeus I, Lundholm K. Protection of metabolic and exercise capacity in unselected weight-losing cancer patients following treatment with recombinant erythropoietin: a randomized prospective study. *Cancer Res* 1998; **58**: 5374-5379
 - 46 **Ludwig H**, Strasser K. Symptomatology of anemia. *Semin Oncol* 2001; **28**: 7-14
 - 47 **Winearls CG**, Oliver DO, Pippard MJ, Reid C, Downing MR, Cotes PM. Effect of human erythropoietin derived from recombinant DNA on the anaemia of patients maintained by chronic haemodialysis. *Lancet* 1986; **2**: 1175-1178
 - 48 **Eschbach JW**, Egrie JC, Downing MR, Browne JK, Adamson JW. Correction of the anemia of end-stage renal disease with recombinant human erythropoietin. Results of a combined phase I and II clinical trial. *N Engl J Med* 1987; **316**: 73-78
 - 49 **Cella D**, Dobrez D, Glaspy J. Control of cancer-related anemia with erythropoietic agents: a review of evidence for improved quality of life and clinical outcomes. *Ann Oncol* 2003; **14**: 511-519
 - 50 **Miyake T**, Kung CK, Goldwasser E. Purification of human erythropoietin. *J Biol Chem* 1977; **252**: 5558-5564
 - 51 **Lin FK**, Suggs S, Lin CH, Browne JK, Smalling R, Egrie JC, Chen KK, Fox GM, Martin F, Stabinsky Z. Cloning and expression of the human erythropoietin gene. *Proc Natl Acad Sci USA* 1985; **82**: 7580-7584
 - 52 **Lacombe C**, Mayeux P. The molecular biology of erythropoietin. *Nephrol Dial Transplant* 1999; **14** Suppl 2: 22-28
 - 53 **Koury MJ**, Bondurant MC. Erythropoietin retards DNA breakdown and prevents programmed death in erythroid progenitor cells. *Science* 1990; **248**: 378-381
 - 54 **Chang J**, Couture F, Young S, McWatters KL, Lau CY. Weekly epoetin alfa maintains hemoglobin, improves quality of life, and reduces transfusion in breast cancer patients receiving chemotherapy. *J Clin Oncol* 2005; **23**: 2597-2605
 - 55 **Witzig TE**, Silberstein PT, Loprinzi CL, Sloan JA, Novotny PJ, Mailliard JA, Rowland KM, Alberts SR, Krook JE, Levitt R, Morton RF. Phase III, randomized, double-blind study of epoetin alfa compared with placebo in anemic patients receiving chemotherapy. *J Clin Oncol* 2005; **23**: 2606-2617
 - 56 **Kaltwasser JP**, Kessler U, Gottschalk R, Stucki G, Möller B. Effect of recombinant human erythropoietin and intravenous iron on anemia and disease activity in rheumatoid arthritis. *J Rheumatol* 2001; **28**: 2430-2436
 - 57 **Egrie JC**, Browne JK. Development and characterization of novel erythropoiesis stimulating protein (NESP). *Br J Cancer* 2001; **84** Suppl 1: 3-10
 - 58 **Macdougall IC**, Gray SJ, Elston O, Breen C, Jenkins B, Browne J, Egrie J. Pharmacokinetics of novel erythropoiesis stimulating protein compared with epoetin alfa in dialysis patients. *J Am Soc Nephrol* 1999; **10**: 2392-2395
 - 59 **Vanrenterghem Y**, Bárány P, Mann JF, Kerr PG, Wilson J, Baker NF, Gray SJ. Randomized trial of darbepoetin alfa for treatment of renal anemia at a reduced dose frequency compared with rHuEPO in dialysis patients. *Kidney Int* 2002; **62**: 2167-2175
 - 60 **Nissenson AR**, Swan SK, Lindberg JS, Soroka SD, Beatey R, Wang C, Picarello N, McDermott-Vitak A, Maroni BJ. Randomized, controlled trial of darbepoetin alfa for the treatment of anemia in hemodialysis patients. *Am J Kidney Dis* 2002; **40**: 110-118
 - 61 **Haselbeck A**, Bailon P, Pahlke W. The discovery and characterization of CERA, an innovative agent for the treatment of anemia. *Blood* 2002; **100**: 227A (Abstract 857)
 - 62 **Macdougall IC**. CERA (Continuous Erythropoietin Receptor Activator): a new erythropoiesis-stimulating agent for the treatment of anemia. *Curr Hematol Rep* 2005; **4**: 436-440
 - 63 **Provenzano R**, Besarab A, Macdougall IC, Dougherty FC, Beyer U on behalf of the BA16528 study group. CERA (Continuous Erythropoietin Receptor Activator) administered up to once every 3 weeks corrects anemia in patients with chronic kidney disease not on dialysis (abstract). *J Am Soc Nephrol* 2004; **15**: 544A
 - 64 **Locatelli F**, Villa G, Arias M, Marchesi D, Dougherty FC, Beyer U on behalf of the BA16286 study group. CERA (Continuous Erythropoietin Receptor Activator) maintains hemoglobin levels in dialysis patients when administered subcutaneously up to once every 4 weeks (abstract). *J Am Soc Nephrol* 2004; **15**: 543A

- 65 **Kochendoerfer GG**, Chen SY, Mao F, Cressman S, Traviglia S, Shao H, Hunter CL, Low DW, Cagle EN, Carnevali M, Gueriguian V, Keogh PJ, Porter H, Stratton SM, Wiedeke MC, Wilken J, Tang J, Levy JJ, Miranda LP, Crnogorac MM, Kalbag S, Botti P, Schindler-Horvat J, Savatski L, Adamson JW, Kung A, Kent SB, Bradburne JA. Design and chemical synthesis of a homogeneous polymer-modified erythropoiesis protein. *Science* 2003; **299**: 884-887
- 66 **Sytkowski AJ**, Lunn ED, Risinger MA, Davis KL. An erythropoietin fusion protein comprised of identical repeating domains exhibits enhanced biological properties. *J Biol Chem* 1999; **274**: 24773-24778
- 67 **Wrighton NC**, Farrell FX, Chang R, Kashyap AK, Barbone FP, Mulcahy LS, Johnson DL, Barrett RW, Jolliffe LK, Dower WJ. Small peptides as potent mimetics of the protein hormone erythropoietin. *Science* 1996; **273**: 458-464
- 68 **Horina JH**, Petritsch W, Schmid CR, Reicht G, Wenzl H, Silly H, Krejs GJ. Treatment of anemia in inflammatory bowel disease with recombinant human erythropoietin: results in three patients. *Gastroenterology* 1993; **104**: 1828-1831
- 69 **Christodoulou DK**, Tsianos EV. Anemia in inflammatory bowel disease - the role of recombinant human erythropoietin. *Eur J Intern Med* 2000; **11**: 222-227
- 70 **Koutroubakis IE**, Karmiris K, Makreas S, Xidakis C, Niniraki M, Kouroumalis EA. Effectiveness of darbepoetin-alfa in combination with intravenous iron sucrose in patients with inflammatory bowel disease and refractory anaemia: a pilot study. *Eur J Gastroenterol Hepatol* 2006; **18**: 421-425
- 71 **Demetri GD**, Kris M, Wade J, Degos L, Cella D. Quality-of-life benefit in chemotherapy patients treated with epoetin alfa is independent of disease response or tumor type: results from a prospective community oncology study. Procrit Study Group. *J Clin Oncol* 1998; **16**: 3412-3425
- 72 **Crawford J**, Cella D, Cleeland CS, Cremieux PY, Demetri GD, Sarokhan BJ, Slavin MB, Glaspy JA. Relationship between changes in hemoglobin level and quality of life during chemotherapy in anemic cancer patients receiving epoetin alfa therapy. *Cancer* 2002; **95**: 888-895
- 73 **Cuzzocrea S**, Mazzon E, Di Paola R, Patel NS, Genovese T, Muià C, De Sarro A, Thiemermann C. Erythropoietin reduces the development of experimental inflammatory bowel disease. *J Pharmacol Exp Ther* 2004; **311**: 1272-1280
- 74 **Fatouros MS**, Vekinis G, Bourantas KL, Mylonakis EP, Scopelitou AS, Malamou-Mitsis VD, Kappas AM. Influence of growth factors erythropoietin and granulocyte macrophage colony stimulating factor on mechanical strength and healing of colonic anastomoses in rats. *Eur J Surg* 1999; **165**: 986-992
- 75 **Bokemeyer C**, Aapro MS, Courdi A, Foubert J, Link H, Osterborg A, Repetto L, Soubeyran P. EORTC guidelines for the use of erythropoietic proteins in anaemic patients with cancer: 2006 update. *Eur J Cancer* 2007; **43**: 258-270
- 76 **Nemeth E**, Rivera S, Gabayan V, Keller C, Taudorf S, Pedersen BK, Ganz T. IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. *J Clin Invest* 2004; **113**: 1271-1276
- 77 **Cronin CC**, Shanahan F. Anemia in patients with chronic inflammatory bowel disease. *Am J Gastroenterol* 2001; **96**: 2296-2298
- 78 **Leyland-Jones B**, Semiglazov V, Pawlicki M, Pienkowski T, Tjulandin S, Manikhas G, Makhson A, Roth A, Dodwell D, Baselga J, Biakhov M, Valuckas K, Voznyi E, Liu X, Vercammen E. Maintaining normal hemoglobin levels with epoetin alfa in mainly nonanemic patients with metastatic breast cancer receiving first-line chemotherapy: a survival study. *J Clin Oncol* 2005; **23**: 5960-5972
- 79 **Besarab A**, Bolton WK, Browne JK, Egrie JC, Nissenson AR, Okamoto DM, Schwab SJ, Goodkin DA. The effects of normal as compared with low hematocrit values in patients with cardiac disease who are receiving hemodialysis and epoetin. *N Engl J Med* 1998; **339**: 584-590
- 80 **Steinbrook R**. Haemoglobin concentrations in chronic kidney disease. *Lancet* 2006; **368**: 2191-2193
- 81 **Casadevall N**, Nataf J, Viron B, Kolta A, Kiladjian JJ, Martin-Dupont P, Michaud P, Papo T, Ugo V, Teyssandier I, Varet B, Mayeux P. Pure red-cell aplasia and antierythropoietin antibodies in patients treated with recombinant erythropoietin. *N Engl J Med* 2002; **346**: 469-475
- 82 **Boven K**, Stryker S, Knight J, Thomas A, van Regenmortel M, Kemeny DM, Power D, Rossert J, Casadevall N. The increased incidence of pure red cell aplasia with an Eprex formulation in uncoated rubber stopper syringes. *Kidney Int* 2005; **67**: 2346-2353
- 83 **Yasuda Y**, Fujita Y, Matsuo T, Koinuma S, Hara S, Tazaki A, Onozaki M, Hashimoto M, Musha T, Ogawa K, Fujita H, Nakamura Y, Shiozaki H, Utsumi H. Erythropoietin regulates tumour growth of human malignancies. *Carcinogenesis* 2003; **24**: 1021-1029
- 84 **Mittelman M**, Neumann D, Peled A, Kanter P, Haran-Ghera N. Erythropoietin induces tumor regression and antitumor immune responses in murine myeloma models. *Proc Natl Acad Sci USA* 2001; **98**: 5181-5186
- 85 **Westenfelder C**, Baranowski RL. Erythropoietin stimulates proliferation of human renal carcinoma cells. *Kidney Int* 2000; **58**: 647-657
- 86 **Acs G**, Acs P, Beckwith SM, Pitts RL, Clements E, Wong K, Verma A. Erythropoietin and erythropoietin receptor expression in human cancer. *Cancer Res* 2001; **61**: 3561-3565
- 87 **Heeschen C**, Aicher A, Lehmann R, Fichtlscherer S, Vasa M, Urbich C, Mildner-Rihm C, Martin H, Zeiher AM, Dimmeler S. Erythropoietin is a potent physiologic stimulus for endothelial progenitor cell mobilization. *Blood* 2003; **102**: 1340-1346
- 88 **D'Haens G**, Geboes K, Rutgeerts P. Endoscopic and histologic healing of Crohn's (ileo-) colitis with azathioprine. *Gastrointest Endosc* 1999; **50**: 667-671
- 89 **D'haens G**, Van Deventer S, Van Hogezaand R, Chalmers D, Kothe C, Baert F, Braakman T, Schaible T, Geboes K, Rutgeerts P. Endoscopic and histological healing with infliximab anti-tumor necrosis factor antibodies in Crohn's disease: A European multicenter trial. *Gastroenterology* 1999; **116**: 1029-1034
- 90 **Hébert PC**, Wells G, Blajchman MA, Marshall J, Martin C, Pagliarello G, Tweeddale M, Schweitzer I, Yetisir E. A multicenter, randomized, controlled clinical trial of transfusion requirements in critical care. Transfusion Requirements in Critical Care Investigators, Canadian Critical Care Trials Group. *N Engl J Med* 1999; **340**: 409-417
- 91 **Lacroix J**, Hébert PC, Hutchison JS, Hume HA, Tucci M, Ducruet T, Gauvin F, Collet JP, Toledano BJ, Robillard P, Joffe A, Biarent D, Meert K, Peters MJ. Transfusion strategies for patients in pediatric intensive care units. *N Engl J Med* 2007; **356**: 1609-1619

S- Editor Ma N L- Editor Anand BS E- Editor Yin DH

Robert Thimme, MD, Professor, Series Editor

Immunity to hepatitis C virus infection: Update 2007

From the editor

This 8-part topic review series, "Hepatitis C virus: virology, immunology and pathogenesis", was designed to provide expert opinion and new insights for the Journal's readership.

Hepatitis C virus (HCV) is a small single-stranded RNA virus that belongs to the *Flaviviridae* virus family. More than 120 million people are chronically HCV infected. The factors that determine the outcome and natural course of HCV infection are not completely understood. However, it is generally accepted that next to virological factors innate and adaptive immune responses play an important role in both, control of HCV infection and as disease pathogenesis (Figure 1).

In recent years, significant progress has been made in understanding basic mechanisms of viral replication, innate and adaptive immune responses, such as CD4⁺ and CD8⁺ T cells as well as neutralizing antibodies, and the possible role of regulatory T cells in orchestrating the adaptive immune response. However, the central question why HCV is able to evade the innate and adaptive immune response and to establish viral persistence in the majority of acutely infected patients is still unknown. In this review series, recent advances

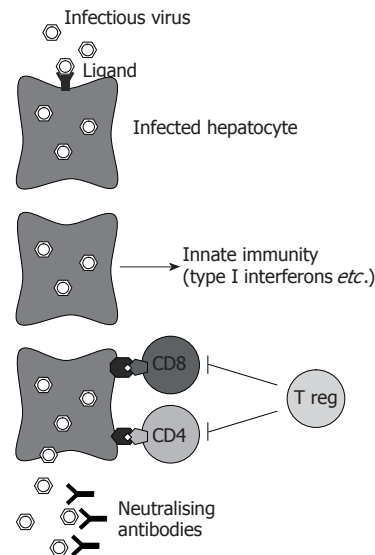


Figure 1 After HCV infection of hepatocytes, innate immune responses are induced. In addition, virally infected cells present antigens to CD4⁺ and CD8⁺ T cells. Both T cell subtypes are regulated by Tregs. Secreted virus can be bound by neutralizing antibodies. All parts of these interactions will be discussed in the following review series.

in HCV virology and immunology will be highlighted. Clearly, a better understanding of the dynamic host-virus interactions during HCV infection is crucial for the development of new prophylactic and therapeutic vaccines.

- 4808 Sequence diversity of HCV: Implications for immune control and therapy
Timm J, Roggendorf M
- 4818 Interaction of hepatitis C virus with the type I interferon system
Weber F
- 4824 Neutralizing antibodies in hepatitis c virus infection
Zeisel MB, Fafi-Kremer S, Fofana I, Barth H, Stoll-Keller F, Doffoël M, Baumert TF
- 4831 CD4⁺ T cell responses in hepatitis C virus infection
Semmo N, Klennerman P
- 4839 Host and viral factors contributing to CD8⁺ T cell failure in hepatitis C virus infection
Neumann-Haefelin C, Spangenberg HC, Blum HE, Thimme R
- 4848 Memory CD8⁺ T cell differentiation in viral infection: A cell for all seasons
Radziejewicz H, Uebelhoer L, Bengsch B, Grakoui A
- 4858 Regulatory T cells in viral hepatitis
Billerbeck E, Böttler T, Thimme R
- 4865 Hepatitis C virus infection and apoptosis
Fischer R, Baumert T, Blum HE

TOPIC HIGHLIGHT

Robert Timme, MD, Professor, Series Editor

Sequence diversity of hepatitis C virus: Implications for immune control and therapy

Joerg Timm, Michael Roggendorf

Joerg Timm, Michael Roggendorf, University of Essen, Institute of Virology, Essen 45122, Germany
Supported by the German Hepatitis Network (BMBF) and the Deutsche Forschungsgemeinschaft
Correspondence to: Joerg Timm, MD, University of Essen, Institute of Virology, Hufelandst 55, Essen 45122, Germany. joerg.timm@uni-due.de
Telephone: +49-201-7232306 Fax: +49-201-7235929
Received: June 26, 2007 Revised: July 9, 2007

Abstract

With approximately 3% of the world's population (170 million people) infected with the hepatitis C virus (HCV), the WHO has declared HCV a global health problem. Upon acute infection about 50%-80% of subjects develop chronic hepatitis with viral persistence being at risk to develop liver cirrhosis and hepatocellular carcinoma. One characteristic of HCV is its enormous sequence diversity, which represents a significant hurdle to the development of both effective vaccines as well as to novel therapeutic interventions. Due to a polymerase that lacks a proofreading function HCV presents with a high rate of evolution, which enables rapid adaptation to a new environment including an activated immune system upon acute infection. Similarly, novel drugs designed to specifically inhibit viral proteins will face the potential problem of rapid selection of drug resistance mutations. This review focuses on the sequence diversity of HCV, the driving forces of evolution and the impact on immune control and treatment response. An important feature of any therapeutic or prophylactic intervention will be an efficient attack of a structurally or functionally important region in the viral protein. The understanding of the driving forces, but also the limits of viral evolution, will be fundamental for the design of novel therapies.

© 2007 WJG. All rights reserved.

Key words: Hepatitis C Virus; Evolution; Escape; Drug resistance; Selection

Timm J, Roggendorf M. Sequence diversity of hepatitis C virus: Implications for immune control and therapy. *World J Gastroenterol* 2007; 13(36): 4808-4817

<http://www.wjgnet.com/1007-9327/13/4808.asp>

INTRODUCTION

With approximately 3% of the world's population (170 million people) infected with the hepatitis C virus (HCV), the WHO has declared HCV a global health problem^[1]. Upon acute infection about 50%-80% of subjects develop chronic hepatitis with viral persistence being at risk to develop liver cirrhosis and hepatocellular carcinoma. The current standard treatment for chronic HCV infection is based on a combination of pegylated interferon alpha and ribavirin^[2,3]. However, this combination is successful in only 50% of all subjects with response rates substantially influenced by the infecting viral genotype. Moreover, the side effects and costs of treatment further limit the use in many regions. Alternative treatment approaches including immunotherapeutic interventions are, therefore, highly warranted. One characteristic of HCV is its enormous sequence diversity, which represents a significant hurdle to the development of both effective vaccines as well as to novel therapeutic interventions. This review focuses on the sequence diversity of HCV, the driving forces of evolution and the impact on immune and control and treatment response.

GENOME STRUCTURE

The HCV genome is an RNA molecule of approximately 9600 nucleotides structured in a coding region that contains one large open reading frame and flanked by non-translated regions at the 5' and 3' ends. The polyprotein is cleaved into structural (core, envelope 1 and 2) and non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) with one additional small protein at the junction between the structural and non-structural elements (p7 protein)^[4]. Until the recent development of an *in vitro* replication system that produces infectious viral particles^[5-7] many of the protein functions have been studied in sub-genomic replication systems or with purified protein after expression.

The 5' NTR forms a highly structured RNA element that contains an internal ribosomal entry site (IRES) that allows interaction with the 40S ribosomal subunit and initiation of cap-independent translation of the viral RNA^[8]. The polyprotein is translated as one large reading frame and subsequently cleaved by host-cellular proteases and virally encoded proteases into the individual proteins^[9]. The structural proteins core, envelope 1 and envelope 2 are cleaved

by host-cellular proteases. Processing of these proteins is believed to take place in a membrane associated complex at the endoplasmic reticulum by signal peptidases^[10]. The core protein forms the viral capsid, binds to the viral RNA and interacts with envelope proteins to form viral particles. Different receptors have been suggested for the interaction of viral particles with the hepatocyte that mediates HCV entry including CD81, scavenger receptor class-B type-I (SR-BI), low-density lipoprotein receptor (LDL), mannose binding lectins (L-SIGN and DC-SIGN) and glycosaminoglycans^[11]. E2 binds with high affinity to the extracellular loop of CD81, a tetraspanin that is expressed on different cell types including hepatocytes^[12]. Cell entry of the virus is CD81 and SR-BI dependent, suggesting that these molecules serve as receptors or co-receptors for infection^[13-16]. However, this interaction does not explain the hepatotropism as CD81 and SR-BI are not exclusively expressed on hepatocytes. Moreover, it has been shown that CD81 and SR-BI are necessary for cell entry of viral particles, but are not sufficient^[16,17]. Some cell lines that express both proteins do not support viral entry. Recently a new candidate has been suggested that may close this gap. Claudin-1, a tight junction component that is highly expressed in the liver, was recently identified as a key factor in the late entry process^[18].

These structural components of HCV are flanked by the non-structural proteins NS2 to NS5B. The function of one additional protein (p7) between these elements remains to be elucidated. It has been suggested that p7 forms an ion channel in planar lipid bilayers^[19,20]. However, it is still unclear whether it is a virion component. NS2 contains an autoprotease, which cleaves the junction between NS2 and NS3^[4]. NS3 is a multifunctional protein with a N-terminal protease domain and a C-terminal RNA helicase/NTPase domain. The NS3 protease cleaves the remaining non-structural proteins with NS4A as a cofactor for this activity. The NS3 RNA helicase/NTPase unwinds RNA and DNA^[21]; however, its role during viral replication is unclear. The integral membrane protein NS4B is sufficient to induce membranous web formation and has been proposed to serve as a scaffold for replication complex assembly. The role of NS5A is again unclear. Numerous protein-protein interactions have been suggested including a role in silencing the host's innate immune response and determining responsiveness to interferon alpha^[22]. Some of these interactions are discussed later. NS5B encodes the viral RNA-dependent RNA polymerase. In the viral replication cycle the positive-strand RNA genome serves as a template to make a negative-strand intermediate, which then again serves as a template to produce multiple nascent genomes. Recently, one additional protein resulting from frameshifted translation of the core protein has been identified (alternative reading frame protein ARFP)^[23]. However, the function of this protein is unknown.

GENOTYPES

Phylogenetic analysis of HCV genomes revealed that sequences fall into different clusters. This observation led to a classification of HCV into different genotypes and a standardized nomenclature was proposed in a consensus

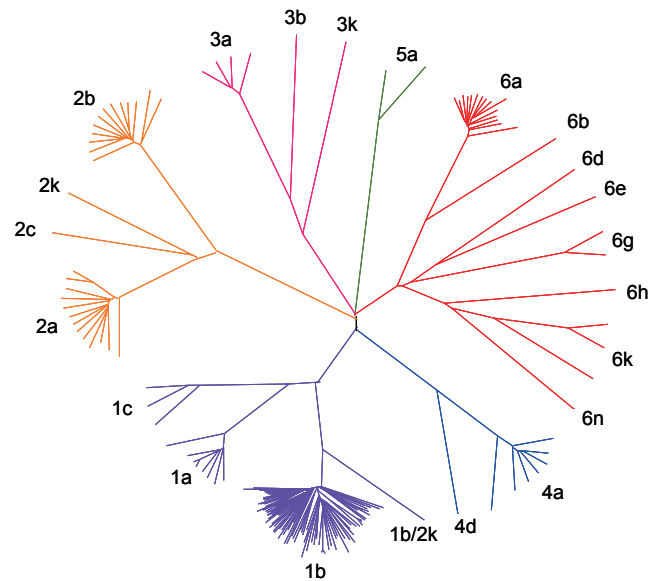


Figure 1 Published HCV Full Genomes. 169 full length HCV sequences available from the Los Alamos National Laboratory (LANL) HCV Sequence Database are illustrated in a phylogenetic tree. HCV sequences fall into six different clusters (genotype 1-6) and are further classified into subtypes. The sequence from one recombinant virus (genotype 1b/2k) is included.

paper in 1994^[24]. The global distribution of HCV genotypes is regionally specific. The predominant genotype in most areas is genotype 1. However, some areas are almost exclusively infected with other genotypes. For example, the predominant genotype in Egypt is genotype 4 and the HCV epidemic in this country could be linked to parenteral treatment of schistosomiasis in the 1950s^[25,26]. In some regions in Africa genotype 2 is more frequent^[27]. In some areas of Asia, however, genotypes 3 and 6 are predominant^[28-30]. Despite substantial sequence variation all genotypes share the same structure of linear genes of nearly identical size. The genotype specific variation of the different genes is remarkably consistent and has enabled many of the currently recognized variants of HCV to be provisionally classified based on partial sequences from subgenomic regions such as core/E1 and NS5B. The original nomenclature was recently updated further standardizing the nomenclature of existing variants^[30]. Based on phylogenetic analysis a classification into 6 major genotypes was proposed and criteria for the designation of new HCV variants were formulated. These proposals provide an HCV nomenclature scheme for the three major public HCV sequence databases (Europe^[31], USA^[32] and Japan: <http://s2as02.genes.nig.ac.jp/>) and eliminate inconsistencies of the current classification procedures. HCV genotypes differ from each other by 31%-33% on the nucleotide level^[33]. The genotypes are further divided into multiple epidemiologically distinct subtypes differing by 20%-25% from one other^[33]. A phylogenetic tree depicting all published complete HCV genomes is presented in Figure 1. For many of the HCV subtypes, particularly for the less frequent ones, complete genome sequences are not available. The lack of sequence data for rare genotypes is profound prompting a sequencing initiative supported by the NIH to improve the HCV sequence databases^[32].

SEQUENCE VARIATION

As a member of the flaviviridae, the virally encoded RNA polymerase of HCV lacks a proof reading function. Replication of this positive-stranded RNA genome is, therefore, characterized by ongoing error rates between 1 in 10000 and 1 in 100000 bp copied, which are typically found for RNA polymerases^[34]. Together with a high turnover rate of estimated 10^{12} virions per day^[35], theoretically every possible mutation in every single position of the genome will be generated in one infected host every day. This high error rate is reflected in the generation of a heterogeneous, but closely related swarm of viruses within the same host referred to as quasispecies. The quasispecies nature of HCV can be best illustrated by sequence analysis of a short, but highly polymorphic region in envelope 2 designated as the hypervariable region 1 (HVR 1). Analysis of clonal sequences reveals that sequences of the viral population from the same subject are highly variable, but still phylogenetically closely related. In public databases normally the consensus sequence, as the most predominant residue at any given position within the quasispecies population, is presented. The quasispecies nature of HCV may have important consequences during a transmission event. Depending on the transmission route the number of transmitted viral RNA copies can be limited and may not represent the true complexity of the sequence diversity of the donor. This bottleneck phenomenon has been described for sexual transmission of HCV^[36] and in the chimpanzee model^[37]. However, the bottleneck could also be interpreted as selection of the optimal strain in the new host during the earliest infection events. Different aspects about the nature of the observed HCV sequence evolution have been published. The next section gives an overview of the different mechanisms.

DRIVING FORCES OF EVOLUTION

Genetic drift

Longitudinal analysis of isolates from subjects with chronic HCV infection calculated a mutation rate on the order of $1.5\text{--}2.0 \times 10^{-3}$ nucleotide substitutions per site per genome per year^[38,39]. In the model of neutral evolution mutations are selectively neutral^[40]. The spread of these neutral mutations is mainly influenced by stochastic factors and is called genetic drift. As a consequence of this stochastic process even disadvantageous mutations can reach fixation when the virus circulates through a sufficiently small population. In turn, advantageous mutations are also affected by genetic drift when they are rare and are occasionally lost from the population. Several studies have described the rapid sequence drift of HCV over time^[33]. The model of neutral evolution and the presumption that such diversification should occur at a constant rate over time provide a framework to estimate times of spread of HCV in specific transmission networks^[41,42]. For example, a recent analysis of viral sequences obtained from an HCV and HIV outbreak in children at the Al-Fateh hospital in Libya, utilizing this molecular clock, demonstrated that the origin of the outbreak predated the year of 1998^[43]. Noteworthy, this analysis excluded the possibility that the

source virus was transmitted by foreign medical staff as suggested by local authorities.

Sequence drift has been suggested in a few studies as the major driving force of HCV evolution. An analysis by Allain *et al* suggested a dominant role in the evolution of envelope 2^[44]. They analyzed clonal sequences of six different transmission pairs years after the transmission event. In this study the ratio of non-synonymous to synonymous mutations in the analyzed region did not support the hypothesis of positive or negative selection. The author's, therefore, concluded that neutral evolution is a major component of the observed sequence diversity. Moreover, the authors did not find a correlation between the strength of the antibody response and the rate of evolution in these patients. However, in this study the strength of the humoral immune response was determined in serum collected years after the transmission event utilizing different HVR 1 peptides corresponding to autologous and heterologous sequences. Therefore, the true antibody response against the virus present during the acute phase of infection might have been underestimated. Another shortcoming of this study is that the inoculum sequence at the time of transmission was unknown and only a single time point years later was available making conclusions about the true evolutionary rate and kinetics difficult.

Positive and negative selection

In contrast to being neutral mutations may also be selected. Many mutations are probably disadvantageous or even deleterious for the virus and these variants are eliminated in a negative selection process. However, some mutations may not have an impact on replication capacity and a few may even be beneficial and confer a replication advantage. Variants harbouring these beneficial mutations will out compete for others in a dynamic process of continuous positive selection. Similar to other highly variable pathogens a complex process of continuous selection has been proposed for HCV^[45]. Theoretically, infections with persistent viruses such as HIV and HCV have time to evolve within the same host before transmission to the next host and may adapt to the specific environment in an individual. The evolution of HCV may, therefore, be substantially influenced by host factors mediating selection pressure on the virus. Even though the consensus sequence may be close to the maximum of viral replication capacity at any one time, the existence of a large and diverse viral population allows rapid, adaptive changes in response to changes in the replication environment. Many variants that are beneficial in a new environment may already be present in a low frequency in the quasispecies population and subsequently out competes the existing dominant sequence. The impact of the quasispecies complexity on the clinical outcome can be profound. Farci *et al* analyzed sequences covering HVR1 obtained during the acute phase of infection from subjects who spontaneously resolved viremia and subjects who continued to chronic infection^[46]. Spontaneous resolution of viremia was predicted by a decrease in quasispecies complexity during the first weeks of infection.

In turn patients with viral persistence had increasing viral diversity suggesting a fast adaptation process to the new environment. A similar effect of quasispecies diversity on the outcome of treatment is discussed^[47,48]. In recent years many studies have been published that aim to characterize the driving forces of this selection process.

Antibodies

The most variable region in the HCV genome is a short fragment spanning 27 amino acids of envelope 2 and is, therefore, designated the hypervariable region 1 (HVR 1). There is strong evidence that the profound sequence diversity in this region is the result of immune pressure by virus specific antibodies. Importantly, there is a close association between the observed sequence diversity in this region and the appearance of HCV specific antibodies in the sera of subjects with acute infection^[49,50]. Patients suffering from common variable immunodeficiency (CVID) who present with hypogammaglobulinemia are not able to produce high titres of HCV specific antibodies and, therefore, are not able to mount humoral immune selection pressure. Analysis of sequences of HVR1 revealed that patients with hypogammaglobulinemia had significantly less amino acid substitutions in this region over time as compared to controls^[51]. In a similar analysis, the rate of non-synonymous and synonymous mutations was compared between core and envelope in patients with and without CVID^[52]. The rate of synonymous or silent mutations was similar in the core and envelope protein. In patients without CVID, as expected, the rate of non-synonymous mutations was much higher in envelope as compared to core, a protein that is known to be highly conserved. However, this high rate of non-synonymous mutations in envelope was not observed in patients with CVID suggesting that evolution is triggered by the presence of anti-HCV antibodies. In the chimpanzee model, it was demonstrated that a high turn-over rate is not sufficient to explain HVR1 sequence diversity. Only minor sequence variation was observed in this region upon serial infection with passage of an infectious HCV clone between 8 different animals^[53]. Noteworthy, samples for the subsequent infection of the next animal were taken during the acute phase before antibodies became detectable. Again, this study indicates that this region in the envelope remains stable in the absence of antibodies despite high level viremia that was present in all animals during the acute phase of infection. Taken together, all these studies suggest that without immune selection pressure only minor sequence changes occur in HVR1.

The lack of an *in vitro* culture system has hampered direct evaluation of these putative escape mechanisms in the envelope protein. Recently, more elegant tools for this type of analysis became available by pseudotyping retroviral particles with HCV glycoproteins (HCVpp)^[54-56]. Utilizing this technique, the impact of neutralizing antibodies on the evolution of HVR1 was demonstrated in a study by von Hahn and co-workers^[57]. Here longitudinal samples were obtained over a time period of 26 years from patient H who was infected in 1977 with genotype 1a. Sera were analyzed for the presence of neutralizing

antibodies against the autologous isolate present at the time of sampling. A neutralizing antibody response could be detected as early as 8 wk after infection against the inoculum strain. Interestingly, the antibodies present in a given sample continuously failed to neutralize HCV pseudoparticles bearing the autologous sequence from the same time point. Longitudinal analyses demonstrate continuous escape from emerging antibodies over the time of infection demonstrating humoral immune pressure as the major driving force for the observed sequence diversity in HVR1.

CD8 T cells

Mutational escape from CD8 T cells targeting viral proteins has been well documented for highly variable pathogens such as HIV and SIV. Similarly, in the chimpanzee model of HCV infection selection of mutations in CD8 epitopes that inhibit recognition by specific T cells has been described by Weiner and co-workers^[58]. In a follow-up analysis the majority of targeted CD8 epitopes in chimpanzees infected with HCV evolved over time and an important role for mutational escape as a contributor for viral persistence has been suggested^[59]. However, acute infection is rarely detected in humans due to lack of specific symptoms making the design of similar longitudinal studies difficult. First evidence for selection pressure by CD8 T cells was obtained from sequence analyses of patients with chronic infection^[60]. Here the T cell response against previously defined CD8 epitopes was determined. In some cases the autologous viral sequence present in the patient differed from the described prototype sequence of the epitope and was not targeted by specific T cell lines derived from that patient. The study included a case where sequence evolution was observed in a follow-up sample suggesting that CD8 escape also plays a role during chronic HCV infection. More recently, several longitudinal studies on patients with acute HCV infection have been published providing compelling evidence for CD8 escape in humans^[61-64]. Probably the most comprehensive analysis was done by Cox *et al.*^[62]. They prospectively followed subjects with ongoing intravenous drug use and high risk behaviour for evidence of acute HCV infection. Using this approach they were able to identify eight patients with acute HCV infection. Samples from these patients were obtained during acute infection (at the time of diagnosis) and after 6 mo. Utilizing comprehensive techniques with overlapping peptides spanning the entire HCV polyprotein the breadth of the immune response was determined. At the same time, sequence evolution between the first and second sample obtained 6 mo later was analyzed. Seventeen of 25 targeted epitopes evolved over time consistent with selection of escape mutations. Of note, the single subject without selection of escape mutations cleared viremia spontaneously. In turn, 50% of the observed sequence changes outside the envelope were associated with a detectable CD8 response. In line with these findings Ray *et al.* analyzed sequences from a single source outbreak infected with HCV genotype 1b and observed reproducible selection of mutations in previously

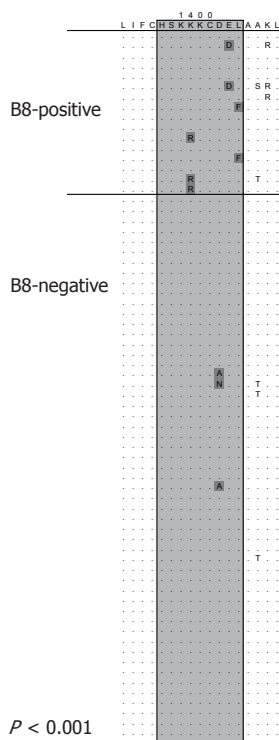


Figure 2 HLA class I-associated sequence polymorphisms in a CD8 epitope. Viral sequences are aligned to a consensus sequence and sorted into sequences derived from HLA B8-positive and HLA B8-negative subjects. Boxed is the region of a described HLA B8-restricted CD8 epitope. Differences from the consensus sequence are significantly more frequent in HLA-B8 positive subjects ($P < 0.001$). This association was reported in [68].

described CD8 epitopes in subjects expressing the restricting HLA-allele^[65]. The observation of widespread escape in individuals during acute HCV infection prompted efforts to determine whether adaptation to HLA class I-restricted selection pressure also occurs at the population level. Moore *et al* analyzed sequences spanning the reverse transcriptase protein of HIV-1 in a large HLA-diverse cohort^[66]. This study revealed accumulation of viral sequence polymorphisms at different sites of the protein in patients sharing the same HLA-allele. Many of these sequence polymorphisms were located inside previously described CD8 epitopes that are restricted by the associated HLA-class I allele. This study demonstrated that MHC class I-associated selection pressure has a major impact on the evolution of HIV-1. A similar analysis was done by the same group in a cohort chronically infected with HCV^[67]. Here, sequences spanning parts of the NS3 protein were analyzed and again a number of associations between sequence polymorphisms and particular HLA-alleles were identified. This analysis was extended to all non-structural proteins in a cohort of 70 subjects with chronic HCV genotype 1a infection with similar results^[68]. An example of an HLA class I-associated sequence polymorphism is illustrated in Figure 2. Viral sequences from all 70 subjects are aligned to a majority consensus sequence and sorted into sequences derived from HLA B8-positive and HLA B8-negative subjects. Boxed is the region of a described HLA B8-restricted CD8 epitope. Differences from the consensus sequence are significantly more frequent in HLA-B8 positive subjects compared to HLA B8-negative subjects indicating that there is reproducible selection pressure on this region in HLA B8-positive subjects. This study included a phylogenetic analysis approach for the detection of HLA-associated sequence polymorphisms highlighting the potential for

false positive detection of such associations by pure statistical approaches. However, these studies suggest that the same evolutionary forces act on HCV and HIV-1 and that selection pressure by virus specific CD8 T cells is an important driver of viral evolution.

CD4 T cells

Spontaneous resolution of viremia after acute infection with HCV has been associated with the emergence of a broad and functionally intact T cell response. There is convincing evidence that HCV specific CD4 T cells are important for viral control in the early phase. However, little is known about mutational escape in targeted CD4 T cell epitopes during HCV infection. In theory, a similar selection process as observed for CD8 T cells could be present. A quasispecies that is mutated inside an immunodominant CD4 epitope may have a selection advantage in an individual with acute HCV infection and could out compete others. Eckels *et al* found evidence for selection of mutations in CD4 epitopes in NS3^[69,70]. Analysis of a large number of clonal sequences revealed a high degree of polymorphisms in regions targeted by CD4 T cells and the ratio of synonymous versus non-synonymous mutations was consistent with positive selection. However, none of the observed variants became the dominant sequence at a second time point 16 mo later. More recently, two studies have described mutational escape from CD4 responses. The first report is part of a vaccination study in the chimpanzee model^[71]. One animal was vaccinated with DNA followed by recombinant vaccinia virus in a prime/boost strategy with HCV NS3 and NS5A/B and subsequently infected with a genotype 1a isolate. After primary infection and transient control of viremia the animal developed chronic infection. Longitudinal sequence analysis of the NS3 and NS5A/B region revealed two non-synonymous mutations. Both of them were located inside regions targeted by CD4 T cells. Additional experiments with synthetic peptides showed that the mutated sequence was not recognized by specific T cells from this animal. A second study focusing on the evolution of the envelope protein in one subject similarly identified amino acid substitutions inside targeted CD4 epitopes consistent with escape^[57]. These findings suggest that CD4 T cells may select mutations during HCV infection; however, the overall extent of CD4 escape as a contributor to the evolution of HCV remains to be clarified.

Innate immunity

Infection with HCV initiates a cascade of events within the infected cell with the goal to generate an antiviral state. The first line of defence builds the innate immune system that is triggered by engagement of pathogen-associated molecular patterns (PAMPs) to specific PAMP receptors^[72]. In case of HCV toll-like receptor 3 (TLR3) and retinoic-acid-inducible gene I (RIG-I)-receptor recognize dsRNA resulting in activation of multiple cellular factors that mediate transcription and secretion of interferon alpha and beta. Engagement of these type I interferons with their cellular receptor activates a series of interferon stimulated genes (ISG) with the goal to initiate an antiviral state with-

in the infected cell. The hepatitis C virus has developed strategies to evade this first line of host immune defence. Recently it was demonstrated that the NS3/4A protease is able to specifically cleave Cardif (CARD adaptor inducing interferon beta) and TRIF (Toll-interleukin-1 receptor domain-containing adaptor-inducing beta interferon)^[73-75]. Both proteins are involved in the activation of interferon regulatory factors (IRFs) and their inactivation, therefore, interferes with the production of interferon alpha and beta. Gale *et al.*^[76] demonstrated *in vitro* that NS5A is able to inhibit protein kinase R (PKR). PKR is activated by interferon alpha and involved in the inhibition of viral RNA translation. Its inhibition, therefore, represents a functional antagonism to the interferon alpha response. Interactions between virus and cellular host factors are sequence specific and negative selection of mutations undermining these immune evasion strategies seems reasonable. However, the underlying mechanisms of the innate immune response are highly conserved in all humans and less dependent on the genetic background of the individual. Therefore, the direction of selection pressure does not change upon transmission to the next host, which makes the observation of positive selection unlikely. Moreover, the interactions between virus and the host's innate immune response take place during the earliest phase of infection making it even more difficult to directly show that viral escape variants are selected.

Drugs

The current standard treatment regimen for patients with chronic hepatitis C is a combination of pegylated interferon alpha with ribavirin^[2,3]. Interestingly, the response rate to this treatment regimen is dependent on the infecting genotype suggesting that sequence differences between genotypes influence the susceptibility to these drugs. Patients infected with genotype 2 and 3 usually show a much faster decline in viral load after initiation of therapy associated with higher sustained response rates. The determinants of this differential responsiveness of different genotypes are poorly understood. Interferon alpha predominantly modulates the immune system^[77]. Engagement of its specific receptor turns on a cascade of IFN-stimulated genes (ISGs) resulting in a non-pathogen specific antiviral state. Different HCV sequences seem to have different capabilities to interfere with this anti-viral strategy. The response rates to treatment dramatically differ not only between different genotypes, but also between isolates of the same subtype. Comparison of sequence isolates that have been successfully treated with isolates that did not respond has put a 40 amino acid stretch of the HCV NS5A protein into the spotlight^[78]. The degree of sequence variation in this region has been associated with treatment outcome and has therefore been designated as the interferon-sensitivity determining region (ISDR). Subsequently, conflicting results have been published in similar studies; however, a meta-analysis supported the impact of this region on treatment outcome^[79]. A correlate of this observation may be the reported inhibitory action of HCV NS5A on PKR^[76]. For this interaction the ISDR and an additional 26 C-terminal amino acid stretch of NS5A are crucial. Therefore, selection of viral variants

during treatment that successfully enhance this interaction seems reasonable. However, neither was selection of mutations observed in the presence of this antiviral drug *in vitro*^[80] nor has selection of variants during treatment in longitudinal studies formally been shown. It remains, therefore, unclear how interferon alpha contributes to evolution.

The exact antiviral mechanisms of ribavirin are even less well established. Several mechanisms have been suggested including inhibition of the HCV polymerase and early chain termination during the replication process. Higher mutation rates in the presence of ribavirin have been reported potentially resulting in an 'error catastrophe'. Two recent studies analyzed the mutation rate in the presence and absence of ribavirin in patients receiving treatment. Hofmann *et al.* analyzed the NS3 and NS5B gene in 14 subjects receiving either ribavirin monotherapy or in combination with interferon alpha^[81]. Based on a comparison of clonal sequences in the quasispecies population before and after initiation of therapy they concluded that the mutation rate of HCV is higher in the presence of ribavirin. These results were reproducible in cell culture with HCV replicon bearing hepatoma cell lines. Even though the overall effect was weak, a dose dependency could be demonstrated and the inactive L-enantiomer did not show the same effect. In a similar analysis by Lutchman *et al.* the mutation rate for NS5B was calculated based on analysis of bulk and clonal sequences of the NS5B gene in 18 subjects receiving ribavirin and 13 subjects receiving placebo^[82]. A significant increase of the mutation rate in the presence of ribavirin was observed after 4 wk of treatment; however, there was no significant difference between the mutation rates after 24 wk compared to the placebo group. The authors of this latter study conclude that ribavirin unlikely acts through an increase of the mutational error rate resulting in an error catastrophe. One study demonstrated selection of a Phe to Tyr mutation in position 415 of the HCV NS5B protein in the presence of ribavirin^[83]. This mutation was associated with a less susceptible phenotype for this drug when tested in the replicon model *in vitro*. This mutation was also observed in 5 out of 16 subjects infected with genotype 1a in the study by Lutchman *et al.* However, it was not reproducible in HCV genotype 1b. It is, therefore, still unclear if specific mutations are selected in the presence of ribavirin.

Future treatment strategies will include small molecules as inhibitors of virus specific protein functions. Drugs like protease and polymerase inhibitors are very successful for the treatment of HIV. However, selection of variants that are resistant to these antiviral compounds is a major challenge in the management of patients infected with HIV. In recent years many compounds have been tested as inhibitors of the HCV protease and polymerase. Some are now available in early clinical trials. The first compound that was tested in humans was the protease inhibitor BILN 2061. In patients infected with genotype 1 the viral load was dramatically decreased after only 2 d of treatment^[84]. However, the drug was designed to inhibit the HCV protease from a genotype 1 isolate with high affinity. As expected, the efficacy was, therefore, much

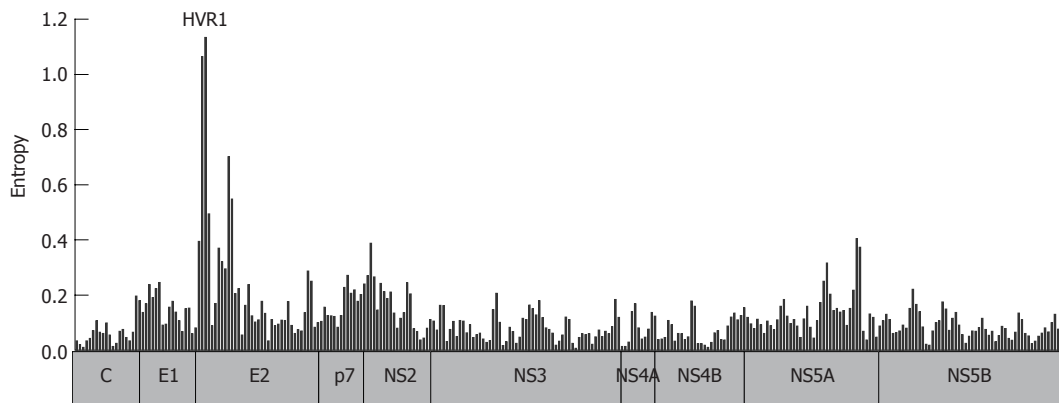


Figure 3 Entropy across the HCV polyprotein. 100 HCV genotype 1b sequences were retrieved from the Los Alamos National Laboratory (LANL) HCV Sequence Database. The entropy score was calculated for 300 windows of 20 residues overlapping by 10 residues utilizing the algorithm implemented in the database.

lower in subjects infected with HCV genotype 2 or 3^[85]. Due to observed toxic effects of this drug in high dosage in animal models further clinical trials were stopped. Other small compounds have meanwhile reached early phases of clinical testing (reviewed in^[86]) including protease inhibitors (VX-950 and SCH 503034) and polymerase inhibitors (NM283 and HCV-796). Along with their proof of excellent efficacy *in vitro* and *in vivo* several reports have been published describing resistance mutations^[87,88]. A recent study by Sarrazin *et al* analyzed clonal sequences from subjects treated with the protease inhibitor VX-950 in a clinical trial^[89]. In this analysis mutations associated with phenotypic resistance were rapidly selected during treatment and the number of resistant clones in each patient correlated well with the virologic response to the drug. The mutations were reproducibly located in only a few positions in the HCV protease gene. Interestingly, the number of resistant clones decreased after cessation of therapy indicating that some mutations are associated with fitness costs and revert back to wild type in the absence of the drug. Future studies will show if combinations of different drugs such as polymerase and protease inhibitors are beneficial to decrease the risk of resistance mutations similar to HIV.

Constraints on sequence diversity-purifying selection

Analysis of available HCV full genome sequences from public databases shows that the degree of sequence variation varies both between different proteins, but also between regions of the same protein (Figure 3). Some regions are highly conserved even across different HCV genotypes. Many of these highly conserved regions represent functionally important motifs in the viral protein in which substantial sequence variation is not tolerated. Viral evolution is clearly limited by structural constraints forcing the virus into a state in which it is able to functionally exist. Many mutations that occur during the replication process are deleterious or disadvantageous to the fitness of the virus and are, therefore, negatively selected. In contrast, as highlighted in this review multiple forces of the immune system or in some cases drugs exact positive selection pressure away from the consensus sequence in the individual. Selection of variants is, therefore, a trade-off between host pressure and functional needs. Purifying selection describes the driving force towards a sequence with optimal replication capacity

in the absence of outside pressure on the virus. Reversion of resistance mutations that have been selected in the presence of antiviral drugs back to the consensus sequence have been first described in the influenza model^[90]. For HIV, reversion of drug resistance mutations is well documented and the concept of a salvage therapy was based on this observation. In this concept treatment is re-initiated after interruption and genotypic reversion of drug resistance. However, the clinical benefit is controversial and with a wide range of antiviral drugs now available for the treatment of HIV this strategy received less attention.

In HCV reversion was first described for an escape mutation that has been selected by virus-specific CTLs^[64]. In this study a virus harbouring an escape mutation in an HLA-B8 restricted epitope in NS3 was transmitted to a host who is HLA-B8 negative and who, therefore, was not able to mount the same T cell response. In the new host the virus continued to evolve back to the prototype sequence and the variant disappeared. Similarly, Ray *et al* analyzed viral sequences from a single source outbreak years after the transmission event^[65]. Interestingly, the source of the virus again had an escape mutation in the same HLA-B8 restricted epitope in NS3. Years later the mutation has reverted back to the prototype sequence in most recipients. Of note, the mutation was stable in subjects who are HLA-B8 positive and who are theoretically able to target this region. Similar to the reversion of CTL escape mutations Sarrazin *et al*^[89] describe in their study on drug resistance to the protease inhibitor VX-960 reversion back to the wild type sequence after treatment was discontinued. These studies illustrate the main selecting forces of HCV evolution. On one side, there is positive selection pressure mainly by the immune system but also in the presence of antiviral drugs. These selection forces are not constant and vary in different hosts and different environments largely depending on the host's genetic background. On the other side, there is negative selection pressure, which presses the virus into a state of optimal replication capacity. This force is more or less constant, but largely depends on the pre-existing sequence configuration such as the genotype or presence of compensatory mutations.

CONCLUSION

The inherent sequence diversity of HCV represents a

major challenge for any treatment. HCV has the ability to rapidly adapt to a hostile environment in the acutely infected individual with an activated immune system at work. An important feature of any therapeutic or prophylactic intervention will be the efficient attack of a structurally or functionally important region in the viral protein. Ideally the attack is targeted against a region that does not tolerate substantial sequence variation in order to limit the ability to evade. Alternatively, a combination of different attacks at different sites may decrease the risk of efficient mutational escape. Recent studies in HIV have suggested that selection of escape mutations may actually be linked to control of viral replication^[91]. Indeed, many of the described CTL escape mutations particularly in the presence of protective HLA-alleles such as HLA-B27 and -B57 as well as some drug resistance mutations in HIV are associated with dramatic fitness costs for the virus. For HCV, little is known about the impact of escape and drug resistance mutations on replication capacity even though their documented reversion in the absence of the drug or immune pressure suggests similar fitness costs. With the recent development of culture systems that include the complete replication cycle we now have the tools at hand to address important questions on the interaction between different selecting forces acting on HCV. The understanding of this interaction is fundamental for the design of novel therapies.

REFERENCES

- 1 **World Health Organization.** Hepatitis C-global prevalence (update). *Weekly Epidemiological Record* 1999; **74**: 425
- 2 **Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Gonçales FL, Häussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Lin A, Hoffman J, Yu J.** Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; **347**: 975-982
- 3 **Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK.** Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; **358**: 958-965
- 4 **Lindenbach BD, Rice CM.** Unravelling hepatitis C virus replication from genome to function. *Nature* 2005; **436**: 933-938
- 5 **Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, Murthy K, Habermann A, Kräusslich HG, Mizokami M, Bartenschlager R, Liang TJ.** Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* 2005; **11**: 791-796
- 6 **Lindenbach BD, Evans MJ, Syder AJ, Wölk B, Tellinghuisen TL, Liu CC, Maruyama T, Hynes RO, Burton DR, McKeating JA, Rice CM.** Complete replication of hepatitis C virus in cell culture. *Science* 2005; **309**: 623-626
- 7 **Zhong J, Gastaminza P, Cheng G, Kapadia S, Kato T, Burton DR, Wieland SF, Uprichard SL, Wakita T, Chisari FV.** Robust hepatitis C virus infection *in vitro*. *Proc Natl Acad Sci USA* 2005; **102**: 9294-9299
- 8 **Spahn CM, Kieft JS, Grassucci RA, Penczek PA, Zhou K, Doudna JA, Frank J.** Hepatitis C virus IRES RNA-induced changes in the conformation of the 40s ribosomal subunit. *Science* 2001; **291**: 1959-1962
- 9 **Lohmann V, Koch JO, Bartenschlager R.** Processing pathways of the hepatitis C virus proteins. *J Hepatol* 1996; **24**: 11-19
- 10 **Liu Q, Tackney C, Bhat RA, Prince AM, Zhang P.** Regulated processing of hepatitis C virus core protein is linked to subcellular localization. *J Virol* 1997; **71**: 657-662
- 11 **Bartosch B, Cosset FL.** Cell entry of hepatitis C virus. *Virology* 2006; **348**: 1-12
- 12 **Pileri P, Uematsu Y, Campagnoli S, Galli G, Falugi F, Petracca R, Weiner AJ, Houghton M, Rosa D, Grandi G, Abrignani S.** Binding of hepatitis C virus to CD81. *Science* 1998; **282**: 938-941
- 13 **Kapadia SB, Barth H, Baumert T, McKeating JA, Chisari FV.** Initiation of hepatitis C virus infection is dependent on cholesterol and cooperativity between CD81 and scavenger receptor B type I. *J Virol* 2007; **81**: 374-383
- 14 **Akazawa D, Date T, Morikawa K, Murayama A, Miyamoto M, Kaga M, Barth H, Baumert TF, Dubuisson J, Wakita T.** CD81 expression is important for the permissiveness of Huh7 cell clones for heterogeneous hepatitis C virus infection. *J Virol* 2007; **81**: 5036-5045
- 15 **Hsu M, Zhang J, Flint M, Logvinoff C, Cheng-Mayer C, Rice CM, McKeating JA.** Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles. *Proc Natl Acad Sci USA* 2003; **100**: 7271-7276
- 16 **Bartosch B, Vitelli A, Granier C, Goujon C, Dubuisson J, Pascale S, Scarselli E, Cortese R, Nicosia A, Cosset FL.** Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor. *J Biol Chem* 2003; **278**: 41624-41630
- 17 **Zhang J, Randall G, Higginbottom A, Monk P, Rice CM, McKeating JA.** CD81 is required for hepatitis C virus glycoprotein-mediated viral infection. *J Virol* 2004; **78**: 1448-1455
- 18 **Evans MJ, von Hahn T, Tscherne DM, Syder AJ, Panis M, Wölk B, Hatzioannou T, McKeating JA, Bieniasz PD, Rice CM.** Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. *Nature* 2007; **446**: 801-805
- 19 **Pavlović D, Neville DC, Argaud O, Blumberg B, Dwek RA, Fischer WB, Zitzmann N.** The hepatitis C virus p7 protein forms an ion channel that is inhibited by long-alkyl-chain iminosugar derivatives. *Proc Natl Acad Sci USA* 2003; **100**: 6104-6108
- 20 **Griffin SD, Beales LP, Clarke DS, Worsfold O, Evans SD, Jaeger J, Harris MP, Rowlands DJ.** The p7 protein of hepatitis C virus forms an ion channel that is blocked by the antiviral drug, Amantadine. *FEBS Lett* 2003; **535**: 34-38
- 21 **Frick DN.** The hepatitis C virus NS3 protein: a model RNA helicase and potential drug target. *Curr Issues Mol Biol* 2007; **9**: 1-20
- 22 **Tellinghuisen TL, Rice CM.** Interaction between hepatitis C virus proteins and host cell factors. *Curr Opin Microbiol* 2002; **5**: 419-427
- 23 **Xu Z, Choi J, Yen TS, Lu W, Strohecker A, Govindarajan S, Chien D, Selby MJ, Ou J.** Synthesis of a novel hepatitis C virus protein by ribosomal frameshift. *EMBO J* 2001; **20**: 3840-3848
- 24 **Simmonds P, Alberti A, Alter HJ, Bonino F, Bradley DW, Brechot C, Brouwer JT, Chan SW, Chayama K, Chen DS.** A proposed system for the nomenclature of hepatitis C viral genotypes. *Hepatology* 1994; **19**: 1321-1324
- 25 **Pybus OG, Drummond AJ, Nakano T, Robertson BH, Rambaut A.** The epidemiology and iatrogenic transmission of hepatitis C virus in Egypt: a Bayesian coalescent approach. *Mol Biol Evol* 2003; **20**: 381-387
- 26 **Ray SC, Arthur RR, Carella A, Bukh J, Thomas DL.** Genetic epidemiology of hepatitis C virus throughout Egypt. *J Infect Dis* 2000; **182**: 698-707
- 27 **Candotti D, Temple J, Sarkodie F, Allain JP.** Frequent recovery and broad genotype 2 diversity characterize hepatitis C virus infection in Ghana, West Africa. *J Virol* 2003; **77**: 7914-7923
- 28 **Wong DA, Tong LK, Lim W.** High prevalence of hepatitis C virus genotype 6 among certain risk groups in Hong Kong. *Eur J Epidemiol* 1998; **14**: 421-426
- 29 **Hissar SS, Goyal A, Kumar M, Pandey C, Suneetha PV, Sood A, Midha V, Sakhuja P, Malhotra V, Sarin SK.** Hepatitis C virus genotype 3 predominates in North and Central India and is associated with significant histopathologic liver disease. *J Med Virol* 2006; **78**: 452-458
- 30 **Simmonds P, Bukh J, Combet C, Deléage G, Enomoto N, Feinstone S, Halfon P, Inchauspé G, Kuiken C, Maertens G, Mizokami M, Murphy DG, Okamoto H, Pawlotsky JM, Penin F, Sablon E, Shin-I T, Stuyver LJ, Thiel HJ, Viazov S, Weiner**

- AJ, Widell A. Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. *Hepatology* 2005; **42**: 962-973
- 31 **Combet C**, Garnier N, Charavay C, Grando D, Crisan D, Lopez J, Dehne-Garcia A, Geourjon C, Bettler E, Hulo C, Le Mercier P, Bartenschlager R, Diepolder H, Moradpour D, Pawlotsky JM, Rice CM, Trépo C, Penin F, Deléage G. euHCVdb: the European hepatitis C virus database. *Nucleic Acids Res* 2007; **35**: D363-D366
- 32 **Kuiken C**, Yusim K, Boykin L, Richardson R. The Los Alamos hepatitis C sequence database. *Bioinformatics* 2005; **21**: 379-384
- 33 **Simmonds P**. Genetic diversity and evolution of hepatitis C virus--15 years on. *J Gen Virol* 2004; **85**: 3173-3188
- 34 **Drake JW**, Charlesworth B, Charlesworth D, Crow JF. Rates of spontaneous mutation. *Genetics* 1998; **148**: 1667-1686
- 35 **Neumann AU**, Lam NP, Dahari H, Gretch DR, Wiley TE, Layden TJ, Perelson AS. Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon-alpha therapy. *Science* 1998; **282**: 103-107
- 36 **Quer J**, Esteban JI, Cos J, Sauleda S, Ocaña L, Martell M, Otero T, Cubero M, Palou E, Murillo P, Esteban R, Guàrdia J. Effect of bottlenecks on evolution of the nonstructural protein 3 gene of hepatitis C virus during sexually transmitted acute resolving infection. *J Virol* 2005; **79**: 15131-15141
- 37 **Nainan OV**, Lu L, Gao FX, Meeks E, Robertson BH, Margolis HS. Selective transmission of hepatitis C virus genotypes and quasispecies in humans and experimentally infected chimpanzees. *J Gen Virol* 2006; **87**: 83-91
- 38 **Rispeter K**, Lu M, Behrens SE, Fumiko C, Yoshida T, Roggendorf M. Hepatitis C virus variability: sequence analysis of an isolate after 10 years of chronic infection. *Virus Genes* 2000; **21**: 179-188
- 39 **Smith DB**, Simmonds P. Characteristics of nucleotide substitution in the hepatitis C virus genome: constraints on sequence change in coding regions at both ends of the genome. *J Mol Evol* 1997; **45**: 238-246
- 40 **Kimura M**. Evolutionary rate at the molecular level. *Nature* 1968; **217**: 624-626
- 41 **Cochrane A**, Searle B, Hardie A, Robertson R, Delahooke T, Cameron S, Tedder RS, Dusheiko GM, De Lamballerie X, Simmonds P. A genetic analysis of hepatitis C virus transmission between injection drug users. *J Infect Dis* 2002; **186**: 1212-1221
- 42 **Pybus OG**, Charleston MA, Gupta S, Rambaut A, Holmes EC, Harvey PH. The epidemic behavior of the hepatitis C virus. *Science* 2001; **292**: 2323-2325
- 43 **de Oliveira T**, Pybus OG, Rambaut A, Salemi M, Cassol S, Ciccozzi M, Rezza G, Gattinara GC, D'Arrigo R, Amicosante M, Perrin L, Colizzi V, Perno CF. Molecular epidemiology: HIV-1 and HCV sequences from Libyan outbreak. *Nature* 2006; **444**: 836-837
- 44 **Allain JP**, Dong Y, Vandamme AM, Moulton V, Salemi M. Evolutionary rate and genetic drift of hepatitis C virus are not correlated with the host immune response: studies of infected donor-recipient clusters. *J Virol* 2000; **74**: 2541-2549
- 45 **Grenfell BT**, Pybus OG, Gog JR, Wood JL, Daly JM, Mumford JA, Holmes EC. Unifying the epidemiological and evolutionary dynamics of pathogens. *Science* 2004; **303**: 327-332
- 46 **Farci P**, Shimoda A, Coiana A, Diaz G, Peddis G, Melpolder JC, Strazzer A, Chien DY, Munoz SJ, Balestrieri A, Purcell RH, Alter HJ. The outcome of acute hepatitis C predicted by the evolution of the viral quasispecies. *Science* 2000; **288**: 339-344
- 47 **Farci P**, Strazzer A, Alter HJ, Farci S, Degioannis D, Coiana A, Peddis G, Usai F, Serra G, Chessa L, Diaz G, Balestrieri A, Purcell RH. Early changes in hepatitis C viral quasispecies during interferon therapy predict the therapeutic outcome. *Proc Natl Acad Sci USA* 2002; **99**: 3081-3086
- 48 **Chambers TJ**, Fan X, Droll DA, Hembrador E, Slater T, Nickells MW, Dustin LB, Dibisceglie AM. Quasispecies heterogeneity within the E1/E2 region as a pretreatment variable during pegylated interferon therapy of chronic hepatitis C virus infection. *J Virol* 2005; **79**: 3071-3083
- 49 **Kato N**, Ootsuyama Y, Sekiya H, Ohkoshi S, Nakazawa T, Hijikata M, Shimotohno K. Genetic drift in hypervariable region 1 of the viral genome in persistent hepatitis C virus infection. *J Virol* 1994; **68**: 4776-4784
- 50 **Weiner AJ**, Geysen HM, Christopherson C, Hall JE, Mason TJ, Saracco G, Bonino F, Crawford K, Marion CD, Crawford KA. Evidence for immune selection of hepatitis C virus (HCV) putative envelope glycoprotein variants: potential role in chronic HCV infections. *Proc Natl Acad Sci USA* 1992; **89**: 3468-3472
- 51 **Booth JC**, Kumar U, Webster D, Monjardino J, Thomas HC. Comparison of the rate of sequence variation in the hypervariable region of E2/NS1 region of hepatitis C virus in normal and hypogammaglobulinemic patients. *Hepatology* 1998; **27**: 223-227
- 52 **Christie JM**, Chapel H, Chapman RW, Rosenberg WM. Immune selection and genetic sequence variation in core and envelope regions of hepatitis C virus. *Hepatology* 1999; **30**: 1037-1044
- 53 **Ray SC**, Mao Q, Lanford RE, Bassett S, Laeyendecker O, Wang YM, Thomas DL. Hypervariable region 1 sequence stability during hepatitis C virus replication in chimpanzees. *J Virol* 2000; **74**: 3058-3066
- 54 **Logvinoff C**, Major ME, Oldach D, Heyward S, Talal A, Balfe P, Feinstone SM, Alter H, Rice CM, McKeating JA. Neutralizing antibody response during acute and chronic hepatitis C virus infection. *Proc Natl Acad Sci USA* 2004; **101**: 10149-10154
- 55 **Drummer HE**, Maerz A, Pountourios P. Cell surface expression of functional hepatitis C virus E1 and E2 glycoproteins. *FEBS Lett* 2003; **546**: 385-390
- 56 **Bartosch B**, Bukh J, Meunier JC, Granier C, Engle RE, Blackwelder WC, Emerson SU, Cosset FL, Purcell RH. In vitro assay for neutralizing antibody to hepatitis C virus: evidence for broadly conserved neutralization epitopes. *Proc Natl Acad Sci USA* 2003; **100**: 14199-14204
- 57 **von Hahn T**, Yoon JC, Alter H, Rice CM, Rehermann B, Balfe P, McKeating JA. Hepatitis C virus continuously escapes from neutralizing antibody and T-cell responses during chronic infection in vivo. *Gastroenterology* 2007; **132**: 667-678
- 58 **Weiner A**, Erickson AL, Kansopon J, Crawford K, Muchmore E, Hughes AL, Houghton M, Walker CM. Persistent hepatitis C virus infection in a chimpanzee is associated with emergence of a cytotoxic T lymphocyte escape variant. *Proc Natl Acad Sci USA* 1995; **92**: 2755-2759
- 59 **Erickson AL**, Kimura Y, Igarashi S, Eichelberger J, Houghton M, Sidney J, McKinney D, Sette A, Hughes AL, Walker CM. The outcome of hepatitis C virus infection is predicted by escape mutations in epitopes targeted by cytotoxic T lymphocytes. *Immunity* 2001; **15**: 883-895
- 60 **Chang KM**, Rehermann B, McHutchison JG, Pasquinelli C, Southwood S, Sette A, Chisari FV. Immunological significance of cytotoxic T lymphocyte epitope variants in patients chronically infected by the hepatitis C virus. *J Clin Invest* 1997; **100**: 2376-2385
- 61 **Guglietta S**, Garbuglia AR, Pacciani V, Scottà C, Perrone MP, Laurenti L, Spada E, Mele A, Capobianchi MR, Taliani G, Folgori A, Vitelli A, Ruggeri L, Nicosia A, Piccolella E, Del Porto P. Positive selection of cytotoxic T lymphocyte escape variants during acute hepatitis C virus infection. *Eur J Immunol* 2005; **35**: 2627-2637
- 62 **Cox AL**, Mosbruger T, Mao Q, Liu Z, Wang XH, Yang HC, Sidney J, Sette A, Pardoll D, Thomas DL, Ray SC. Cellular immune selection with hepatitis C virus persistence in humans. *J Exp Med* 2005; **201**: 1741-1752
- 63 **Tester I**, Smyk-Pearson S, Wang P, Wertheimer A, Yao E, Lewinsohn DM, Tavis JE, Rosen HR. Immune evasion versus recovery after acute hepatitis C virus infection from a shared source. *J Exp Med* 2005; **201**: 1725-1731
- 64 **Timm J**, Lauer GM, Kavanagh DG, Sheridan I, Kim AY, Lucas M, Pillay T, Ouchi K, Reyrol LL, Schulze zur Wiesch J, Gandhi RT, Chung RT, Bhardwaj N, Klenerman P, Walker BD, Allen TM. CD8 epitope escape and reversion in acute HCV infection. *J Exp Med* 2004; **200**: 1593-1604
- 65 **Ray SC**, Fanning L, Wang XH, Netski DM, Kenny-Walsh

- E, Thomas DL. Divergent and convergent evolution after a common-source outbreak of hepatitis C virus. *J Exp Med* 2005; **201**: 1753-1759
- 66 **Moore CB**, John M, James IR, Christiansen FT, Witt CS, Mallal SA. Evidence of HIV-1 adaptation to HLA-restricted immune responses at a population level. *Science* 2002; **296**: 1439-1443
- 67 **Gaudieri S**, Rauch A, Park LP, Freitas E, Herrmann S, Jeffrey G, Cheng W, Pfafferoth K, Naidoo K, Chapman R, Battegay M, Weber R, Telenti A, Furrer H, James I, Lucas M, Mallal SA. Evidence of viral adaptation to HLA class I-restricted immune pressure in chronic hepatitis C virus infection. *J Virol* 2006; **80**: 11094-11104
- 68 **Timm J**, Li B, Daniels MG, Bhattacharya T, Reyrol LL, Allgaier R, Kuntzen T, Fischer W, Nolan BE, Duncan J, Schulze Zur Wiesch J, Kim AY, Frahm N, Brander C, Chung RT, Lauer GM, Korber BT, Allen TM. Human leukocyte antigen-associated sequence polymorphisms in hepatitis C virus reveal reproducible immune responses and constraints on viral evolution. *Hepatology* 2007; **46**: 339-349
- 69 **Eckels DD**, Zhou H, Bian TH, Wang H. Identification of antigenic escape variants in an immunodominant epitope of hepatitis C virus. *Int Immunol* 1999; **11**: 577-583
- 70 **Wang H**, Eckels DD. Mutations in immunodominant T cell epitopes derived from the nonstructural 3 protein of hepatitis C virus have the potential for generating escape variants that may have important consequences for T cell recognition. *J Immunol* 1999; **162**: 4177-4183
- 71 **Puig M**, Mihalik K, Tilton JC, Williams O, Merchlinsky M, Connors M, Feinstone SM, Major ME. CD4+ immune escape and subsequent T-cell failure following chimpanzee immunization against hepatitis C virus. *Hepatology* 2006; **44**: 736-745
- 72 **Gale M**, Foy EM. Evasion of intracellular host defence by hepatitis C virus. *Nature* 2005; **436**: 939-945
- 73 **Li K**, Foy E, Ferreon JC, Nakamura M, Ferreon AC, Ikeda M, Ray SC, Gale M, Lemon SM. Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. *Proc Natl Acad Sci USA* 2005; **102**: 2992-2997
- 74 **Breiman A**, Grandvaux N, Lin R, Ottone C, Akira S, Yoneyama M, Fujita T, Hiscott J, Meurs EF. Inhibition of RIG-I-dependent signaling to the interferon pathway during hepatitis C virus expression and restoration of signaling by IKKepsilon. *J Virol* 2005; **79**: 3969-3978
- 75 **Meylan E**, Curran J, Hofmann K, Moradpour D, Binder M, Bartenschlager R, Tschopp J. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature* 2005; **437**: 1167-1172
- 76 **Gale M**, Blakely CM, Kwiciszewski B, Tan SL, Dossett M, Tang NM, Korth MJ, Polyak SJ, Gretsch DR, Katze MG. Control of PKR protein kinase by hepatitis C virus nonstructural 5A protein: molecular mechanisms of kinase regulation. *Mol Cell Biol* 1998; **18**: 5208-5218
- 77 **Feld JJ**, Hoofnagle JH. Mechanism of action of interferon and ribavirin in treatment of hepatitis C. *Nature* 2005; **436**: 967-972
- 78 **Chayama K**, Tsubota A, Kobayashi M, Okamoto K, Hashimoto M, Miyano Y, Koike H, Kobayashi M, Koida I, Arase Y, Saitoh S, Suzuki Y, Murashima N, Ikeda K, Kumada H. Pretreatment virus load and multiple amino acid substitutions in the interferon sensitivity-determining region predict the outcome of interferon treatment in patients with chronic genotype 1b hepatitis C virus infection. *Hepatology* 1997; **25**: 745-749
- 79 **Pascu M**, Martus P, Höhne M, Wiedenmann B, Hopf U, Schreier E, Berg T. Sustained virological response in hepatitis C virus type 1b infected patients is predicted by the number of mutations within the NS5A-ISDR: a meta-analysis focused on geographical differences. *Gut* 2004; **53**: 1345-1351
- 80 **Aus dem Siepen M**, Oniangue-Ndza C, Wiese M, Ross S, Roggendorf M, Viazov S. Interferon-alpha and ribavirin resistance of Huh7 cells transfected with HCV subgenomic replicon. *Virus Res* 2007; **125**: 109-113
- 81 **Hofmann WP**, Polta A, Herrmann E, Mihm U, Kronenberger B, Sonntag T, Lohmann V, Schönberger B, Zeuzem S, Sarrazin C. Mutagenic effect of ribavirin on hepatitis C nonstructural 5B quasispecies *in vitro* and during antiviral therapy. *Gastroenterology* 2007; **132**: 921-930
- 82 **Lutchman G**, Danehower S, Song BC, Liang TJ, Hoofnagle JH, Thomson M, Ghany MG. Mutation rate of the hepatitis C virus NS5B in patients undergoing treatment with ribavirin monotherapy. *Gastroenterology* 2007; **132**: 1757-1766
- 83 **Young KC**, Lindsay KL, Lee KJ, Liu WC, He JW, Milstein SL, Lai MM. Identification of a ribavirin-resistant NS5B mutation of hepatitis C virus during ribavirin monotherapy. *Hepatology* 2003; **38**: 869-878
- 84 **Hinrichsen H**, Benhamou Y, Wedemeyer H, Reiser M, Sentjens RE, Calleja JL, Fornis X, Erhardt A, Crönlein J, Chaves RL, Yong CL, Nehmiz G, Steinmann GG. Short-term antiviral efficacy of BILN 2061, a hepatitis C virus serine protease inhibitor, in hepatitis C genotype 1 patients. *Gastroenterology* 2004; **127**: 1347-1355
- 85 **Reiser M**, Hinrichsen H, Benhamou Y, Reesink HW, Wedemeyer H, Avendano C, Riba N, Yong CL, Nehmiz G, Steinmann GG. Antiviral efficacy of NS3-serine protease inhibitor BILN-2061 in patients with chronic genotype 2 and 3 hepatitis C. *Hepatology* 2005; **41**: 832-835
- 86 **Pawlotsky JM**, Chevaliez S, McHutchison JG. The hepatitis C virus life cycle as a target for new antiviral therapies. *Gastroenterology* 2007; **132**: 1979-1998
- 87 **Lin C**, Lin K, Luong YP, Rao BG, Wei YY, Brennan DL, Fulghum JR, Hsiao HM, Ma S, Maxwell JP, Cottrell KM, Perni RB, Gates CA, Kwong AD. In vitro resistance studies of hepatitis C virus serine protease inhibitors, VX-950 and BILN 2061: structural analysis indicates different resistance mechanisms. *J Biol Chem* 2004; **279**: 17508-17514
- 88 **Tong X**, Chase R, Skelton A, Chen T, Wright-Minogue J, Malcolm BA. Identification and analysis of fitness of resistance mutations against the HCV protease inhibitor SCH 503034. *Antiviral Res* 2006; **70**: 28-38
- 89 **Sarrazin C**, Kieffer TL, Bartels D, Hanzelka B, Müh U, Welker M, Wincheringer D, Zhou Y, Chu HM, Lin C, Weegink C, Reesink H, Zeuzem S, Kwong AD. Dynamic hepatitis C virus genotypic and phenotypic changes in patients treated with the protease inhibitor telaprevir. *Gastroenterology* 2007; **132**: 1767-1777
- 90 **Grambas S**, Bennett MS, Hay AJ. Influence of amantadine resistance mutations on the pH regulatory function of the M2 protein of influenza A viruses. *Virology* 1992; **191**: 541-549
- 91 **Altfeld M**, Allen TM. Hitting HIV where it hurts: an alternative approach to HIV vaccine design. *Trends Immunol* 2006; **27**: 504-510

S- Editor Ma N L- Editor Rippe RA E- Editor Ma WH

TOPIC HIGHLIGHT

Robert Thimme, MD, Professor, Series Editor

Interaction of hepatitis C virus with the type I interferon system

Friedemann Weber

Friedemann Weber, Abteilung Virologie, Institut für Medizinische Mikrobiologie und Hygiene, Universität Freiburg, Freiburg D-79008, Germany

Supported by the Deutsche Forschungsgemeinschaft

Correspondence to: Friedemann Weber, Abteilung Virologie, Institut für Medizinische Mikrobiologie und Hygiene, Universität Freiburg, Freiburg D-79008, Germany. friedemann.weber@uniklinik-freiburg.de

Telephone: +49-761-2036614 Fax: +49-761-2036562

Received: June 26, 2007 Revised: July 9, 2007

Abstract

Hepatitis C virus (HCV) needs to tightly manipulate host defences in order to establish infection. The innate immune response slows down viral replication by activating cytokines such as the type I interferons (IFN- α / β), which trigger the synthesis of antiviral proteins and modulate the adaptive immune system. HCV has therefore developed a number of countermeasures to stay ahead of the IFN system. Here, I will attempt to summarize the current state of research regarding IFN responses against HCV and the viral escape strategies. Particular emphasis will be put on the newly discovered mechanisms HCV employs to avoid the induction of IFN in infected cells.

© 2007 WJG. All rights reserved.

Key words: Hepatitis C virus; Innate immunity; Interferon system; Escape mechanisms

Weber F. Interaction of hepatitis C virus with the type I interferon system. *World J Gastroenterol* 2007; 13(36): 4818-4823

<http://www.wjgnet.com/1007-9327/13/4818.asp>

INTRODUCTION

The type I interferon system which mainly involves IFN- α and - β is a powerful and universal intracellular defence system against viruses. Knockout mice which are unresponsive to IFN- α / β due to targeted deletions in the type I IFN receptor quickly succumb to viral infections although they have a regular adaptive immune system^[1,2].

Likewise, humans with genetic defects in STAT-1, which is involved in the signaling cascade of the IFN system, die of viral disease at an early age^[3].

INTERFERON INDUCTION

All nucleated cells of the mammalian body are able to synthesize and secrete type I IFNs in response to virus infection. Secreted IFNs are then recognized by neighboring cells and cause them to express potent antiviral proteins^[4,5]. As a result, virus multiplication is slowed down or even stopped, and the organism buys time for the establishment of an adaptive immune response.

Type I IFNs are classified according to their amino acid sequence and comprise a large number (at least 13) of IFN- α subtypes and a single IFN- β ^[6], as well as some additional family members^[7,8]. Expression patterns, i.e. which IFNs will be synthesized at which time point, mostly depend on the particular cell type.

Fibroblasts secrete mainly IFN- β as an initial response to infection but switch to IFN- α during the subsequent amplification phase of the IFN response^[9]. By contrast, dendritic cells, which play an important role in immunosurveillance, directly secrete high levels of IFN- α subtypes^[10,11].

Induction of IFN- β gene expression in fibroblasts occurs by the intracellular, so-called “classic pathway” (Figure 1). In infected cells, a signaling chain is activated by viral RNA molecules which are generated during genome transcription and replication^[12]. Two intracellular RNA helicases, RIG-I^[13] and MDA5^[14], act as sentinels for viral RNA^[15-17]. Then, a recently discovered adaptor protein binds to RIG-I and MDA5 and mediates the signal to downstream factors. It is called either Cardif for “CARD adaptor inducing IFN- β ”^[18], IPS-1 for “interferon- β -promoter stimulator 1”^[19], MAVS for “mitochondrial antiviral signaling” molecule^[20], or VISA for “virus-induced signaling adaptor”^[21]. Cardif/IPS-1/MAVS/VISA activates two I κ B kinase (IKK)-related kinases, IKK ϵ and TANK-binding kinase-1 (TBK-1), which phosphorylate the transcription factor IRF-3^[22,23]. IRF-3 is a member of the IFN regulatory factor (IRF) family and plays a central role in the activation of the IFN- β promoter^[24]. Phosphorylated IRF-3 homo-dimerizes and moves into the nucleus where it recruits the transcriptional coactivators p300 and CREB-binding protein (CBP) to

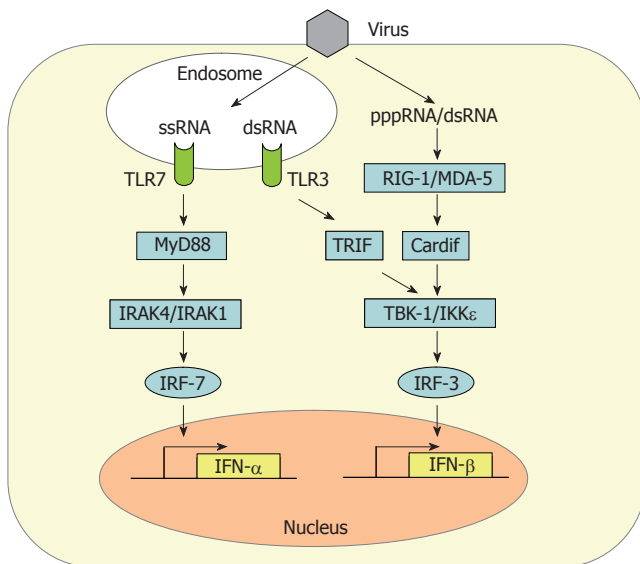


Figure 1 Type I IFN gene expression. Detection of viral ssRNA and dsRNA leads to transactivation of IFN- α and IFN- β promoters by IRF-7 and IRF-3. IRF-3 is phosphorylated by the kinases IKK ϵ and TBK-1 which in turn are activated by the intracellular RNA-sensor proteins RIG-I and MDA5. RIG-I preferentially senses 5'triphosphorylated ssRNAs (pppRNA) whereas MDA-5 recognizes dsRNA. Cardif (also termed IPS-1/MAVS/VISA) serves as an adaptor protein connecting RNA sensing and IRF-3 phosphorylation. A second dsRNA signaling pathway involves endosomal TLR-3 and the adaptor protein TRIF which also activates IKK ϵ and TBK-1. The endosomal ssRNA receptor TLR7 utilizes the adaptor protein MyD88 to stimulate IFN- α synthesis via the kinases IRAK4 and IRAK1 and the transcription factor IRF-7.

initiate IFN- β mRNA synthesis^[24,25]. This first-wave IFN triggers expression of a related factor, IRF-7, which in fibroblasts is only present in low amounts^[26]. IRF-7 can be activated the same way as IRF-3^[27-29], leading to a positive-feedback loop that initiates the synthesis of several IFN- α subtypes as the second-wave IFNs^[9,30]. In addition, NF- κ B and AP-1 are recruited in a dsRNA-dependent way^[31,32]. Together these transcription factors strongly upregulate IFN- β gene expression.

Until very recently, it was assumed that the main trigger of intracellular cytokine induction by all viruses is double-stranded RNA (dsRNA) which supposedly forms as a by-product of genome replication. However, we have recently found that some viruses do not produce substantial amounts of dsRNA^[33]. Instead, ssRNA containing a 5' triphosphate group is much more potent than dsRNA in activating RIG-I-dependent IFN induction^[34-36].

Among the cells of the lymphatic system, myeloid dendritic cells (mDCs)^[11] and, most prominently, plasmacytoid dendritic cells (pDCs)^[10] are the main IFN producers. In addition to the classical, intracellular pathway of IFN induction described above, pDCs sense the presence of viruses by the extracytoplasmic toll-like receptors (TLRs)^[37-39]. It is thought that TLRs serve as sensors for viral infection of phagocytosed cells^[40]. Human pDCs mostly express TLR7 and TLR9 which recognize viral single-stranded(ss) RNA and dsDNA, respectively^[41], whereas mDCs express TLR3 which responds to dsRNA^[42]. Upon activation, TLRs signal through different intracellular adaptor molecules such as

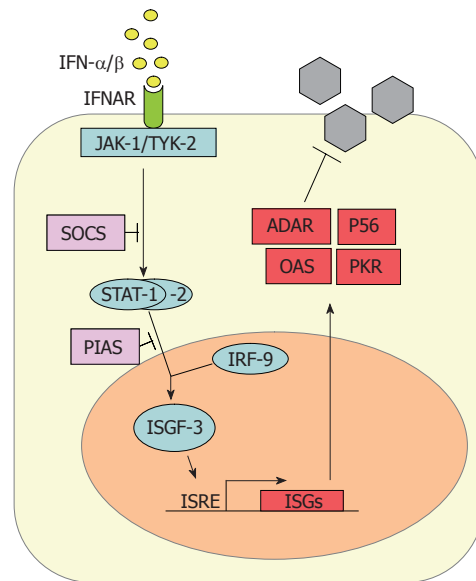


Figure 2 Cellular response to IFNs. Newly synthesized IFN- α/β binds to its cognate receptor (IFNAR) and activates the expression of numerous IFN-stimulated genes (ISGs) via the JAK/STAT pathway. ADAR, P56, OAS and PKR are IFN-stimulated gene products with antiviral properties against HCV. The SOCS and PIAS proteins negatively regulate the IFN-induced signaling pathway at different stages.

MyD88 (TLR7 and 9) or TRIF (TLR3) to induce IFN transcription^[41]. Interestingly, DCs already contain high levels of IRF-7^[43,44], thus explaining their ability to rapidly produce high amounts of alpha-IFNs. Furthermore, TLR7 and TLR9 are retained in the endosomes of pDCs to allow prolonged IFN induction signaling^[45].

INTERFERON SIGNALING

IFN- α/β subtypes all bind to and activate a common type I IFN receptor. It consists of two subunits (IFNAR-1 and IFNAR-2) and is present on virtually all host cells^[5,6]. Binding of IFN- α/β leads to heterodimerization of the IFNAR subunits and to conformational changes in the intracellular parts of the receptor which activate the so-called JAK-STAT signaling pathway (Figure 2). The signal transducer and activator of transcription (STAT) proteins are latent cytoplasmic transcription factors which become phosphorylated by the Janus kinase (JAK) family members JAK-1 and TYK-2^[46]. Phosphorylated STAT-1 and STAT-2 recruit a third factor, IRF-9 (also called p48), to form a complex known as IFN stimulated gene factor 3 (ISGF-3). The ISGF-3 heterotrimer translocates to the nucleus and binds to IFN-stimulated response elements (ISRE) in the promoter regions of IFN-stimulated genes (ISGs), thereby inducing their transcription.

Several specialized proteins serve as negative regulators and inhibitors of the JAK-STAT pathway. For example, the suppressor of cytokine signaling (SOCS) proteins specifically prevent STAT activation by binding to activated cytokine receptors, inhibiting the activity of JAKs, and targeting bound signaling proteins for proteasomal degradation^[47]. Also, the protein inhibitor of activated STAT (PIAS) family members function as small

ubiquitin-like modifier (SUMO) E3 ligases and inhibit the transcriptional activity of STATs^[48].

INTERFERON EFFECTOR PROTEINS WITH ANTIVIRAL ACTIVITY AGAINST HCV

IFN- α combined with ribavirin is the standard treatment for HCV infection, and its effect can be potentiated by co-administration of IFN- γ ^[49,50]. IFN- α/β activates the expression of more than 300 IFN-stimulated genes (ISGs) which have antiviral, antiproliferative, and immunomodulatory functions^[51,52]. IFN-induced proteins include enzymes, transcription factors, cell surface glycoproteins, cytokines, chemokines and a large number of factors that need to be further characterized. Up to now, only a few antiviral proteins have been characterized in detail. Type I IFNs are known to be effective against HCV replicon systems^[53,54], and several IFN-induced proteins have documented anti-HCV activity, namely protein kinase R (PKR)^[55], the RNA-specific adenosine deaminase 1 (ADAR 1)^[56], the 2'-5' oligoadenylate synthetases (2-5 OAS) / RNaseL system^[57], and P56^[58].

PKR, ADAR1, and 2-5 OAS are constitutively expressed in normal cells in a latent, inactive form. Basal mRNA levels are upregulated by IFN- α/β and these enzymes need to be activated by viral dsRNA. PKR is a serine-threonine kinase that phosphorylates the α subunit of the eukaryotic translation initiation factor eIF2^[59]. As a consequence, translation of cellular and viral mRNAs is blocked. ADAR 1 catalyzes the deamination of adenosine on target dsRNAs to yield inosine. As a result the secondary structure is destabilized due to a change from an AU base pair to the less stable IU base pair and mutations accumulate within the viral genome^[5]. The 2-5 OAS catalyzes the synthesis of short 2'-5' oligoadenylates that activate the latent endoribonuclease RNaseL^[60]. RNaseL, in turn, then degrades both viral and cellular RNAs, leading to viral inhibition^[61]. P56 binds the eukaryotic initiation factor 3e (eIF3e) subunit of the eukaryotic translation initiation factor eIF3. It functions as an inhibitor of translation initiation at the level of eIF3 ternary complex formation and is likely to suppress viral RNA translation^[62,63].

INTERACTION WITH INNATE IMMUNE RESPONSES

Several recent studies have clarified that the RNA of HCV is a potent trigger of IFN induction, leading to the establishment of an antiviral state. Therefore, in order to establish infection and to persist in the human host, HCV has been forced to evolve efficient counterstrategies. Intracellular IFN induction by HCV appears to be mostly mediated by RIG-I binding to viral RNA^[64]. Extracellularly, no specific TLR has been identified yet, but by deduction from data on related flaviviruses, TLR3 and TLR7 would be the most obvious candidates. The dsRNA-binding TLR3 was shown to be activated by West Nile virus^[65], and the ssRNA-binding TLR7 is activated by Dengue virus^[66]. Moreover, TLR7 can elicit HCV immunity, and a synthetic

TLR7 agonist reduced HCV mRNA and protein levels in HuH-7 hepatocytes^[67]. It is important to note that TLR7 is expressed in hepatocytes of normal as well as HCV-infected people^[67]. Thus, TLR7 may indeed play a role during natural infection.

On the other hand, HCV is capable of disturbing the IFN response at multiple levels^[68,69]. With respect to IFN induction, it was recently discovered that the NS3/4A protease specifically cleaves Cardif^[418] as well as TRIF^[70,71]. Since both these adaptor proteins are important for IFN induction via the classical intracellular pathway (Cardif) and the TLR3-driven endosomal pathway (TRIF), NS3/4A is the key factor of HCV to disturb IRF-3 activation^[72] which would otherwise result in IFN gene transcription. In addition, NS3 directly interacts with TBK1 to inhibit its association with IRF-3 and its activation^[73].

With respect to the IFN response, it was shown that expression of the full-length virus genome or the core protein suppresses IFN signal transduction^[74,75]. Most likely, this is due to an up-regulation of protein phosphatase 2A by ER stress^[76], resulting in association of STAT1 with its inhibitor PIAS1^[77]. Moreover, for the core protein it was shown that it interferes with the JAK/STAT pathway^[78] and is able to activate the JAK-STAT signaling inhibitor SOCS-3^[79], further contributing to the HCV-induced block of IFN signaling.

HCV also directly counteracts the antiviral IFN response. The NS5A protein, which confers a multitude of functions in virus replication^[80], also plays a key role in escape from the antiviral action of IFN. A stretch of 40 amino acids on NS5A, termed the IFN sensitivity region (ISDR), was correlated with responsiveness to IFN therapy^[81-83]. Moreover, NS5A was shown to directly bind to and repress PKR, and this interaction involved the ISDR^[84]. However, other groups did not find a connection between viral IFN susceptibility and a particular ISDR sequence^[85-87], and PKR activity was not affected by expression of the HCV genome^[88] or NS5A^[89], although NS5A clearly reduced the antiviral effects of IFN^[89]. A possible solution for this discrepancy could be that ISDR sequence variations affect the efficiency of HCV replication^[90,91]. Thus, the correlation between particular ISDR sequences and IFN sensitivity could be caused by differences in HCV replication strength. In addition, NS5A induces IL-8 (also termed CXCL-8), a chemokine which inhibits the antiviral actions of IFN^[92]. Elevated IL-8 levels were indeed detected in the sera of IFN non-responders^[93]. Moreover, in cell culture CXCL-8 protein levels are positively associated with chronic HCV replication and CXCL-8 removal inhibits HCV replication^[94]. Interestingly, CXCL-8 cannot only be induced by NS5A, but also by the HCV RNA-sensitive RIG-I pathway^[95].

NS5A also interferes with the 2-5 OAS/RNaseL pathway by binding to 2-5 OAS^[96]. Furthermore, the HCV genome sequences of IFN-resistant strains have fewer RNase L recognition sites than those of more IFN-sensitive ones^[97], thus allowing escape from nucleolytic cleavage^[97]. PKR activity is also modified by the internal ribosome entry site (IRES) of HCV^[98] and the E2 protein^[99].

The multiple countermeasures of HCV to avoid a

fully-fledged IFN response appear to be quite efficient, since 85% of the HCV-infected patients develop a chronic infection, and up to 60% of those patients do not respond to IFN therapy or experience a relapse when therapy is stopped^[100]. Our rapidly increasing knowledge about HCV immune escape will certainly lead to a significant improvement in both prevention and therapy for hepatitis C.

REFERENCES

- Müller U, Steinhoff U, Reis LF, Hemmi S, Pavlovic J, Zinkernagel RM, Aguet M. Functional role of type I and type II interferons in antiviral defense. *Science* 1994; **264**: 1918-1921
- Weber F, Kochs G, Haller O. Inverse interference: how viruses fight the interferon system. *Viral Immunol* 2004; **17**: 498-515
- Dupuis S, Jouanguy E, Al-Hajjar S, Fieschi C, Al-Mohsen IZ, Al-Jumaah S, Yang K, Chapgier A, Eidenschenk C, Eid P, Al Ghonaium A, Tufenkeji H, Frayha H, Al-Gazlan S, Al-Rayes H, Schreiber RD, Gresser I, Casanova JL. Impaired response to interferon-alpha/beta and lethal viral disease in human STAT1 deficiency. *Nat Genet* 2003; **33**: 388-391
- Haller O, Kochs G, Weber F. The interferon response circuit: induction and suppression by pathogenic viruses. *Virology* 2006; **344**: 119-130
- Samuel CE. Antiviral actions of interferons. *Clin Microbiol Rev* 2001; **14**: 778-809, table of contents
- Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD. How cells respond to interferons. *Annu Rev Biochem* 1998; **67**: 227-264
- Roberts RM, Ezashi T, Rosenfeld CS, Ealy AD, Kubisch HM. Evolution of the interferon tau genes and their promoters, and maternal-trophoblast interactions in control of their expression. *Reprod Suppl* 2003; **61**: 239-251
- van Pesch V, Lanaya H, Renauld JC, Michiels T. Characterization of the murine alpha interferon gene family. *J Virol* 2004; **78**: 8219-8228
- Marié I, Durbin JE, Levy DE. Differential viral induction of distinct interferon-alpha genes by positive feedback through interferon regulatory factor-7. *EMBO J* 1998; **17**: 6660-6669
- Colonna M, Krug A, Cella M. Interferon-producing cells: on the front line in immune responses against pathogens. *Curr Opin Immunol* 2002; **14**: 373-379
- Diebold SS, Montoya M, Unger H, Alexopoulou L, Roy P, Haswell LE, Al-Shamkhani A, Flavell R, Borrow P, Reis e Sousa C. Viral infection switches non-plasmacytoid dendritic cells into high interferon producers. *Nature* 2003; **424**: 324-328
- Bowie AG, Fitzgerald KA. RIG-I: tri-ling to discriminate between self and non-self RNA. *Trends Immunol* 2007; **28**: 147-150
- Yoneyama M, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, Miyagishi M, Taira K, Akira S, Fujita T. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat Immunol* 2004; **5**: 730-737
- Andrejeva J, Childs KS, Young DF, Carlos TS, Stock N, Goodbourn S, Randall RE. The V proteins of paramyxoviruses bind the IFN-inducible RNA helicase, mda-5, and inhibit its activation of the IFN-beta promoter. *Proc Natl Acad Sci USA* 2004; **101**: 17264-17269
- Kato H, Sato S, Yoneyama M, Yamamoto M, Uematsu S, Matsui K, Tsujimura T, Takeda K, Fujita T, Takeuchi O, Akira S. Cell type-specific involvement of RIG-I in antiviral response. *Immunity* 2005; **23**: 19-28
- Yoneyama M, Kikuchi M, Matsumoto K, Imaizumi T, Miyagishi M, Taira K, Foy E, Loo YM, Gale M, Akira S, Yonehara S, Kato A, Fujita T. Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. *J Immunol* 2005; **175**: 2851-2858
- Yoneyama M, Fujita T. Function of RIG-I-like receptors in antiviral innate immunity. *J Biol Chem* 2007; **282**: 15315-15318
- Meylan E, Curran J, Hofmann K, Moradpour D, Binder M, Bartenschlager R, Tschopp J. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature* 2005; **437**: 1167-1172
- Kawai T, Takahashi K, Sato S, Coban C, Kumar H, Kato H, Ishii KJ, Takeuchi O, Akira S. IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nat Immunol* 2005; **6**: 981-988
- Seth RB, Sun L, Ea CK, Chen ZJ. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. *Cell* 2005; **122**: 669-682
- Xu LG, Wang YY, Han KJ, Li LY, Zhai Z, Shu HB. VISA is an adapter protein required for virus-triggered IFN-beta signaling. *Mol Cell* 2005; **19**: 727-740
- Fitzgerald KA, McWhirter SM, Faia KL, Rowe DC, Latz E, Golenbock DT, Coyle AJ, Liao SM, Maniatis T. IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. *Nat Immunol* 2003; **4**: 491-496
- Sharma S, tenOever BR, Grandvaux N, Zhou GP, Lin R, Hiscott J. Triggering the interferon antiviral response through an IKK-related pathway. *Science* 2003; **300**: 1148-1151
- Hiscott J. Triggering the innate antiviral response through IRF-3 activation. *J Biol Chem* 2007; **282**: 15325-15329
- Suhara W, Yoneyama M, Kitabayashi I, Fujita T. Direct involvement of CREB-binding protein/p300 in sequence-specific DNA binding of virus-activated interferon regulatory factor-3 holocomplex. *J Biol Chem* 2002; **277**: 22304-22313
- Sato M, Suemori H, Hata N, Asagiri M, Ogasawara K, Nakao K, Nakaya T, Katsuki M, Noguchi S, Tanaka N, Taniguchi T. Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-alpha/beta gene induction. *Immunity* 2000; **13**: 539-548
- Iwamura T, Yoneyama M, Yamaguchi K, Suhara W, Mori W, Shiota K, Okabe Y, Namiki H, Fujita T. Induction of IRF-3/-7 kinase and NF-kappaB in response to double-stranded RNA and virus infection: common and unique pathways. *Genes Cells* 2001; **6**: 375-388
- tenOever BR, Sharma S, Zou W, Sun Q, Grandvaux N, Julkunen I, Hemmi H, Yamamoto M, Akira S, Yeh WC, Lin R, Hiscott J. Activation of TBK1 and IKKvarepsilon kinases by vesicular stomatitis virus infection and the role of viral ribonucleoprotein in the development of interferon antiviral immunity. *J Virol* 2004; **78**: 10636-10649
- Smith EJ, Marié I, Prakash A, Garcia-Sastre A, Levy DE. IRF3 and IRF7 phosphorylation in virus-infected cells does not require double-stranded RNA-dependent protein kinase R or Ikappa B kinase but is blocked by Vaccinia virus E3L protein. *J Biol Chem* 2001; **276**: 8951-8957
- Sato M, Hata N, Asagiri M, Nakaya T, Taniguchi T, Tanaka N. Positive feedback regulation of type I IFN genes by the IFN-inducible transcription factor IRF-7. *FEBS Lett* 1998; **441**: 106-110
- Chu WM, Ostertag D, Li ZW, Chang L, Chen Y, Hu Y, Williams B, Perrault J, Karin M. JNK2 and IKKbeta are required for activating the innate response to viral infection. *Immunity* 1999; **11**: 721-731
- Yang YL, Reis LF, Pavlovic J, Aguzzi A, Schäfer R, Kumar A, Williams BR, Aguet M, Weissmann C. Deficient signaling in mice devoid of double-stranded RNA-dependent protein kinase. *EMBO J* 1995; **14**: 6095-6106
- Weber F, Wagner V, Rasmussen SB, Hartmann R, Paludan SR. Double-stranded RNA is produced by positive-strand RNA viruses and DNA viruses but not in detectable amounts by negative-strand RNA viruses. *J Virol* 2006; **80**: 5059-5064
- Hornung V, Ellegast J, Kim S, Brzózka K, Jung A, Kato H, Poeck H, Akira S, Conzelmann KK, Schlee M, Endres S, Hartmann G. 5'-Triphosphate RNA is the ligand for RIG-I. *Science* 2006; **314**: 994-997
- Pichlmair A, Schulz O, Tan CP, Näslund TI, Liljeström P, Weber F, Reis e Sousa C. RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. *Science* 2006; **314**: 997-1001
- Plumet S, Herschke F, Bourhis JM, Valentin H, Longhi

- S, Gerlier D. Cytosolic 5'-triphosphate ended viral leader transcript of measles virus as activator of the RIG I-mediated interferon response. *PLoS One* 2007; **2**: e279
- 37 **Beutler B**. Inferences, questions and possibilities in Toll-like receptor signalling. *Nature* 2004; **430**: 257-263
 - 38 **Bowie AG**, Haga IR. The role of Toll-like receptors in the host response to viruses. *Mol Immunol* 2005; **42**: 859-867
 - 39 **Uematsu S**, Akira S. Toll-like receptors and Type I interferons. *J Biol Chem* 2007; **282**: 15319-15323
 - 40 **Schulz O**, Diebold SS, Chen M, Näslund TI, Nolte MA, Alexopoulou L, Azuma YT, Flavell RA, Liljeström P, Reis e Sousa C. Toll-like receptor 3 promotes cross-priming to virus-infected cells. *Nature* 2005; **433**: 887-892
 - 41 **Iwasaki A**, Medzhitov R. Toll-like receptor control of the adaptive immune responses. *Nat Immunol* 2004; **5**: 987-995
 - 42 **Alexopoulou L**, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* 2001; **413**: 732-738
 - 43 **Kerkmann M**, Rothenfusser S, Hornung V, Towarowski A, Wagner M, Sarris A, Giese T, Endres S, Hartmann G. Activation with CpG-A and CpG-B oligonucleotides reveals two distinct regulatory pathways of type I IFN synthesis in human plasmacytoid dendritic cells. *J Immunol* 2003; **170**: 4465-4474
 - 44 **Prakash A**, Smith E, Lee CK, Levy DE. Tissue-specific positive feedback requirements for production of type I interferon following virus infection. *J Biol Chem* 2005; **280**: 18651-18657
 - 45 **Honda K**, Ohba Y, Yanai H, Negishi H, Mizutani T, Takaoka A, Taya C, Taniguchi T. Spatiotemporal regulation of MyD88-IRF-7 signalling for robust type-I interferon induction. *Nature* 2005; **434**: 1035-1040
 - 46 **Levy DE**, Darnell JE. Stats: transcriptional control and biological impact. *Nat Rev Mol Cell Biol* 2002; **3**: 651-662
 - 47 **Kubo M**, Hanada T, Yoshimura A. Suppressors of cytokine signaling and immunity. *Nat Immunol* 2003; **4**: 1169-1176
 - 48 **Shuai K**, Liu B. Regulation of gene-activation pathways by PIAS proteins in the immune system. *Nat Rev Immunol* 2005; **5**: 593-605
 - 49 **Katayama K**, Kasahara A, Sasaki Y, Kashiwagi T, Naito M, Masuzawa M, Katoh M, Yoshihara H, Kamada T, Mukuda T, Hijioka T, Hori M, Hayashi N. Immunological response to interferon-gamma priming prior to interferon-alpha treatment in refractory chronic hepatitis C in relation to viral clearance. *J Viral Hepat* 2001; **8**: 180-185
 - 50 **Okuse C**, Rinaudo JA, Farrar K, Wells F, Korba BE. Enhancement of antiviral activity against hepatitis C virus in vitro by interferon combination therapy. *Antiviral Res* 2005; **65**: 23-34
 - 51 **de Veer MJ**, Holko M, Frevel M, Walker E, Der S, Paranjape JM, Silverman RH, Williams BR. Functional classification of interferon-stimulated genes identified using microarrays. *J Leukoc Biol* 2001; **69**: 912-920
 - 52 **Der SD**, Zhou A, Williams BR, Silverman RH. Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. *Proc Natl Acad Sci USA* 1998; **95**: 15623-15628
 - 53 **Guo JT**, Bichko VV, Seeger C. Effect of alpha interferon on the hepatitis C virus replicon. *J Virol* 2001; **75**: 8516-8523
 - 54 **Frese M**, Pietschmann T, Moradpour D, Haller O, Bartenschlager R. Interferon-alpha inhibits hepatitis C virus subgenomic RNA replication by an MxA-independent pathway. *J Gen Virol* 2001; **82**: 723-733
 - 55 **Pflugheber J**, Fredericksen B, Sumpter R, Wang C, Ware F, Sodora DL, Gale M. Regulation of PKR and IRF-1 during hepatitis C virus RNA replication. *Proc Natl Acad Sci USA* 2002; **99**: 4650-4655
 - 56 **Taylor DR**, Puig M, Darnell ME, Mihalik K, Feinstone SM. New antiviral pathway that mediates hepatitis C virus replicon interferon sensitivity through ADAR1. *J Virol* 2005; **79**: 6291-6298
 - 57 **Guo JT**, Sohn JA, Zhu Q, Seeger C. Mechanism of the interferon alpha response against hepatitis C virus replicons. *Virology* 2004; **325**: 71-81
 - 58 **Wang C**, Pflugheber J, Sumpter R Jr, Sodora DL, Hui D, Sen GC, Gale M Jr. Alpha interferon induces distinct translational control programs to suppress hepatitis C virus RNA replication. *J Virol* 2003; **77**: 3898-3912
 - 59 **Williams BR**. PKR; a sentinel kinase for cellular stress. *Oncogene* 1999; **18**: 6112-6120
 - 60 **Silverman RH**. Fascination with 2-5A-dependent RNase: a unique enzyme that functions in interferon action. *J Interferon Res* 1994; **14**: 101-104
 - 61 **Zhou A**, Paranjape J, Brown TL, Nie H, Naik S, Dong B, Chang A, Trapp B, Fairchild R, Colmenares C, Silverman RH. Interferon action and apoptosis are defective in mice devoid of 2',5'-oligoadenylate-dependent RNase L. *EMBO J* 1997; **16**: 6355-6363
 - 62 **Hui DJ**, Bhasker CR, Merrick WC, Sen GC. Viral stress-inducible protein p56 inhibits translation by blocking the interaction of eIF3 with the ternary complex eIF2.GTP.Met-tRNAi. *J Biol Chem* 2003; **278**: 39477-39482
 - 63 **Terenzi F**, Pal S, Sen GC. Induction and mode of action of the viral stress-inducible murine proteins, P56 and P54. *Virology* 2005; **340**: 116-124
 - 64 **Sumpter R**, Loo YM, Foy E, Li K, Yoneyama M, Fujita T, Lemon SM, Gale M. Regulating intracellular antiviral defense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I. *J Virol* 2005; **79**: 2689-2699
 - 65 **Wang T**, Town T, Alexopoulou L, Anderson JF, Fikrig E, Flavell RA. Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. *Nat Med* 2004; **10**: 1366-1373
 - 66 **Wang JP**, Liu P, Latz E, Golenbock DT, Finberg RW, Libraty DH. Flavivirus activation of plasmacytoid dendritic cells delineates key elements of TLR7 signaling beyond endosomal recognition. *J Immunol* 2006; **177**: 7114-7121
 - 67 **Lee J**, Wu CC, Lee KJ, Chuang TH, Katakura K, Liu YT, Chan M, Tawatao R, Chung M, Shen C, Cottam HB, Lai MM, Raz E, Carson DA. Activation of anti-hepatitis C virus responses via Toll-like receptor 7. *Proc Natl Acad Sci USA* 2006; **103**: 1828-1833
 - 68 **Gale M Jr**, Foy EM. Evasion of intracellular host defence by hepatitis C virus. *Nature* 2005; **436**: 939-945
 - 69 **Thimme R**, Lohmann V, Weber F. A target on the move: innate and adaptive immune escape strategies of hepatitis C virus. *Antiviral Res* 2006; **69**: 129-141
 - 70 **Breiman A**, Grandvaux N, Lin R, Ottone C, Akira S, Yoneyama M, Fujita T, Hiscott J, Meurs EF. Inhibition of RIG-I-dependent signaling to the interferon pathway during hepatitis C virus expression and restoration of signaling by IKKepsilon. *J Virol* 2005; **79**: 3969-3978
 - 71 **Li K**, Foy E, Ferreón JC, Nakamura M, Ferreón AC, Ikeda M, Ray SC, Gale M, Lemon SM. Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. *Proc Natl Acad Sci USA* 2005; **102**: 2992-2997
 - 72 **Foy E**, Li K, Wang C, Sumpter R, Ikeda M, Lemon SM, Gale M. Regulation of interferon regulatory factor-3 by the hepatitis C virus serine protease. *Science* 2003; **300**: 1145-1148
 - 73 **Otsuka M**, Kato N, Moriyama M, Taniguchi H, Wang Y, Dharel N, Kawabe T, Omata M. Interaction between the HCV NS3 protein and the host TBK1 protein leads to inhibition of cellular antiviral responses. *Hepatology* 2005; **41**: 1004-1012
 - 74 **Heim MH**, Moradpour D, Blum HE. Expression of hepatitis C virus proteins inhibits signal transduction through the Jak-STAT pathway. *J Virol* 1999; **73**: 8469-8475
 - 75 **Melén K**, Fagerlund R, Nyqvist M, Keskinen P, Julkunen I. Expression of hepatitis C virus core protein inhibits interferon-induced nuclear import of STATs. *J Med Virol* 2004; **73**: 536-547
 - 76 **Christen V**, Treves S, Duong FH, Heim MH. Activation of endoplasmic reticulum stress response by hepatitis viruses up-regulates protein phosphatase 2A. *Hepatology* 2007; **46**: 558-565
 - 77 **Duong FH**, Filipowicz M, Tripodi M, La Monica N, Heim MH. Hepatitis C virus inhibits interferon signaling through up-regulation of protein phosphatase 2A. *Gastroenterology* 2004;

- 126: 263-277
- 78 **de Lucas S**, Bartolome J, Carreno V. Hepatitis C virus core protein down-regulates transcription of interferon-induced antiviral genes. *J Infect Dis* 2005; **191**: 93-99
 - 79 **Bode JG**, Ludwig S, Ehrhardt C, Albrecht U, Erhardt A, Schaper F, Heinrich PC, Häussinger D. IFN- α antagonistic activity of HCV core protein involves induction of suppressor of cytokine signaling-3. *FASEB J* 2003; **17**: 488-490
 - 80 **Macdonald A**, Harris M. Hepatitis C virus NS5A: tales of a promiscuous protein. *J Gen Virol* 2004; **85**: 2485-2502
 - 81 **Enomoto N**, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Izumi N, Marumo F, Sato C. Comparison of full-length sequences of interferon-sensitive and resistant hepatitis C virus 1b. Sensitivity to interferon is conferred by amino acid substitutions in the NS5A region. *J Clin Invest* 1995; **96**: 224-230
 - 82 **Enomoto N**, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Ogura Y, Izumi N, Marumo F, Sato C. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. *N Engl J Med* 1996; **334**: 77-81
 - 83 **Witherell GW**, Beineke P. Statistical analysis of combined substitutions in nonstructural 5A region of hepatitis C virus and interferon response. *J Med Virol* 2001; **63**: 8-16
 - 84 **Gale M**, Blakely CM, Kwieciszewski B, Tan SL, Dossett M, Tang NM, Korth MJ, Polyak SJ, Gretch DR, Katze MG. Control of PKR protein kinase by hepatitis C virus nonstructural 5A protein: molecular mechanisms of kinase regulation. *Mol Cell Biol* 1998; **18**: 5208-5218
 - 85 **Aizaki H**, Saito S, Ogino T, Miyajima N, Harada T, Matsuura Y, Miyamura T, Kohase M. Suppression of interferon-induced antiviral activity in cells expressing hepatitis C virus proteins. *J Interferon Cytokine Res* 2000; **20**: 1111-1120
 - 86 **Aus dem Siepen M**, Lohmann V, Wiese M, Ross S, Roggendorf M, Viazov S. Nonstructural protein 5A does not contribute to the resistance of hepatitis C virus replication to interferon α in cell culture. *Virology* 2005; **336**: 131-136
 - 87 **Paterson M**, Laxton CD, Thomas HC, Ackrill AM, Foster GR. Hepatitis C virus NS5A protein inhibits interferon antiviral activity, but the effects do not correlate with clinical response. *Gastroenterology* 1999; **117**: 1187-1197
 - 88 **François C**, Duverlie G, Rebouillat D, Khorsi H, Castelain S, Blum HE, Gatignol A, Wychowski C, Moradpour D, Meurs EF. Expression of hepatitis C virus proteins interferes with the antiviral action of interferon independently of PKR-mediated control of protein synthesis. *J Virol* 2000; **74**: 5587-5596
 - 89 **Podevin P**, Sabile A, Gajardo R, Delhem N, Abadie A, Lozach PY, Beretta L, Bréchet C. Expression of hepatitis C virus NS5A natural mutants in a hepatocytic cell line inhibits the antiviral effect of interferon in a PKR-independent manner. *Hepatology* 2001; **33**: 1503-1511
 - 90 **Blight KJ**, Kolykhalov AA, Rice CM. Efficient initiation of HCV RNA replication in cell culture. *Science* 2000; **290**: 1972-1974
 - 91 **Appel N**, Pietschmann T, Bartenschlager R. Mutational analysis of hepatitis C virus nonstructural protein 5A: potential role of differential phosphorylation in RNA replication and identification of a genetically flexible domain. *J Virol* 2005; **79**: 3187-3194
 - 92 **Polyak SJ**, Khabar KS, Paschal DM, Ezelle HJ, Duverlie G, Barber GN, Levy DE, Mukaida N, Gretch DR. Hepatitis C virus nonstructural 5A protein induces interleukin-8, leading to partial inhibition of the interferon-induced antiviral response. *J Virol* 2001; **75**: 6095-6106
 - 93 **Polyak SJ**, Khabar KS, Rezeiq M, Gretch DR. Elevated levels of interleukin-8 in serum are associated with hepatitis C virus infection and resistance to interferon therapy. *J Virol* 2001; **75**: 6209-6211
 - 94 **Koo BC**, McPoland P, Wagoner JP, Kane OJ, Lohmann V, Polyak SJ. Relationships between hepatitis C virus replication and CXCL-8 production in vitro. *J Virol* 2006; **80**: 7885-7893
 - 95 **Wagoner J**, Austin M, Green J, Imaizumi T, Casola A, Brasier A, Khabar KS, Wakita T, Gale M, Polyak SJ. Regulation of CXCL-8 (interleukin-8) induction by double-stranded RNA signaling pathways during hepatitis C virus infection. *J Virol* 2007; **81**: 309-318
 - 96 **Taguchi T**, Nagano-Fujii M, Akutsu M, Kadoya H, Ohgimoto S, Ishido S, Hotta H. Hepatitis C virus NS5A protein interacts with 2',5'-oligoadenylate synthetase and inhibits antiviral activity of IFN in an IFN sensitivity-determining region-independent manner. *J Gen Virol* 2004; **85**: 959-969
 - 97 **Han JQ**, Barton DJ. Activation and evasion of the antiviral 2' -5' oligoadenylate synthetase/ribonuclease L pathway by hepatitis C virus mRNA. *RNA* 2002; **8**: 512-525
 - 98 **Vyas J**, Elia A, Clemens MJ. Inhibition of the protein kinase PKR by the internal ribosome entry site of hepatitis C virus genomic RNA. *RNA* 2003; **9**: 858-870
 - 99 **Taylor DR**, Shi ST, Romano PR, Barber GN, Lai MM. Inhibition of the interferon-inducible protein kinase PKR by HCV E2 protein. *Science* 1999; **285**: 107-110
 - 100 **Pawlotsky JM**. The nature of interferon- α resistance in hepatitis C virus infection. *Curr Opin Infect Dis* 2003; **16**: 587-592

S- Editor Ma N L- Editor Roberts SE E- Editor Ma WH

TOPIC HIGHLIGHT

Robert Thimme, MD, Professor, Series Editor

Neutralizing antibodies in hepatitis C virus infection

Mirjam B Zeisel, Samira Fafi-Kremer, Isabel Fofana, Heidi Barth, Françoise Stoll-Keller, Michel Doffoël, Thomas F Baumert

Mirjam B Zeisel, Samira Fafi-Kremer, Isabel Fofana, Françoise Stoll-Keller, Thomas F Baumert, Inserm, U748, Strasbourg, France

Mirjam B Zeisel, Samira Fafi-Kremer, Isabel Fofana, Françoise Stoll-Keller, Michel Doffoël, Thomas F Baumert, Université Louis Pasteur, Strasbourg, France

Michel Doffoël, Thomas F Baumert, Service d'Hépatogastro-entérologie, Hôpitaux Universitaires de Strasbourg; Strasbourg, France

Heidi Barth, Liver Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD, United States

Supported by Inserm, France; Université Louis Pasteur, France; the European Union (Virgil Network of Excellence); the Deutsche Forschungsgemeinschaft (Ba1417/11-1), Germany; the ANR chair of excellence program and ANRS, France; Inserm "Poste Vert" research fellowship in the framework of Inserm European Associated Laboratory Inserm U748-Department of Medicine II, University of Freiburg, Germany

Correspondence to: Thomas F Baumert, MD, Inserm Unité 748, Université Louis Pasteur, 3 Rue Koeberlé, Strasbourg F-67000, France. thomas.baumert@viro-ulp.u-strasbg.fr

Telephone: +33-390-243702 Fax: +33-390-243723

Received: June 26, 2007 Revised: July 9, 2007

Abstract

Hepatitis C virus (HCV) is a major cause of hepatitis world-wide. The majority of infected individuals develop chronic hepatitis which can then progress to liver cirrhosis and hepatocellular carcinoma. Spontaneous viral clearance occurs in about 20%-30% of acutely infected individuals and results in resolution of infection without sequelae. Both viral and host factors appear to play an important role for resolution of acute infection. A large body of evidence suggests that a strong, multispecific and long-lasting cellular immune response appears to be important for control of viral infection in acute hepatitis C. Due to the lack of convenient neutralization assays, the impact of neutralizing responses for control of viral infection had been less defined. In recent years, the development of robust tissue culture model systems for HCV entry and infection has finally allowed study of antibody-mediated neutralization and to gain further insights into viral targets of host neutralizing responses. In addition, detailed analysis of antibody-mediated neutralization in individual patients as well as cohorts with well defined viral isolates has enabled the study of neutralizing responses in the course of HCV infection and characterization of the impact of neutralizing antibodies

for control of viral infection. This review will summarize recent progress in the understanding of the molecular mechanisms of antibody-mediated neutralization and its impact for HCV pathogenesis.

© 2007 The WJG Press. All rights reserved.

Key words: Hepatitis C virus; Virus-host cell interaction; Viral entry; Neutralizing antibodies

Zeisel MB, Fafi-Kremer S, Fofana I, Barth H, Stoll-Keller F, Doffoël M, Baumert TF. Neutralizing antibodies in hepatitis C virus infection. *World J Gastroenterol* 2007; 13(36): 4824-4830

<http://www.wjgnet.com/1007-9327/13/4824.asp>

INTRODUCTION

With an estimated 170 million infected individuals, hepatitis C virus (HCV) has a major impact on public health^[1]. HCV is a major cause of hepatitis world-wide. The majority of infected individuals develop chronic hepatitis which can then progress to liver cirrhosis and hepatocellular carcinoma. Treatment options for chronic HCV infection are limited and a vaccine to prevent HCV infection is not available.

HCV is a small enveloped positive-strand RNA virus that belongs to the genus *Hepacivirus* of the *Flaviviridae* family. This virus exhibits high genetic heterogeneity and has been classified into six genotypes and several subtypes. The HCV genome encodes a single precursor polypeptide of about 3000 amino acids that is cleaved co- and post-translationally by host and viral proteases into functional structural and non-structural proteins. The virion is composed of three different structural proteins: The core protein forming the viral nucleocapsid and two envelope glycoproteins, E1 and E2.

In vivo, HCV infects only humans and chimpanzees^[2]. Each individual is infected with a mixture of distinct but closely related HCV genomes, termed quaspecies. The liver is the primary target organ of HCV, and the hepatocyte is its primary target cell. Replication of the HCV genome has been demonstrated *in vivo* and *in vitro* in liver hepatocytes, and hematopoietic cells including dendritic cells and B lymphocytes^[3,4]. HCV establishes persistent infection in the majority of infected individuals

despite the fact that it is recognized and targeted by the host's immune system^[5].

Viral proteins are recognized as non-self by the host's immune system and induce the production of antibodies. During the natural course of infection, a large number of antibodies targeting epitopes of both structural and non-structural proteins are produced. The vast majority of antibodies induced have no antiviral activity, either because they are elicited by intracellular, degraded or incompletely processed proteins released from dying cells or because they are directed against epitopes that do not play any role in the virus entry process^[6,7]. A small proportion of antibodies termed "neutralizing antibodies" are able to target exposed epitopes of the viral structural proteins and neutralize the infectious virus by preventing or controlling viral infection. This review will summarize the current knowledge about host neutralizing responses in HCV infection. It starts with a brief description of the current model systems allowing the study of neutralizing responses, followed by viral targets of neutralizing antibodies. Finally, neutralizing responses in the course of HCV infection and the impact of neutralizing antibodies for HCV pathogenesis are discussed.

MODEL SYSTEMS FOR THE STUDY OF ANTIBODY-MEDIATED VIRUS NEUTRALIZATION

For many years, studies of host neutralizing responses against HCV had been hampered by the lack of a convenient tissue culture system for HCV entry and infection. In recent years, several *in vitro* models have been developed to study defined aspects of HCV host cell interaction and antibody-mediated virus neutralization: These include recombinant HCV envelope glycoproteins^[8,9], HCV-like particles^[10], HCV pseudotyped particles^[11-13], and, more recently, cell-culture derived infectious HCV^[14-16]. Recombinant HCV envelope glycoproteins have been successfully used as a surrogate model to study virus-host cell interaction leading to the identification of putative HCV receptor candidates including CD81^[8], scavenger receptor class B type I (SR-BI)^[9] and heparan sulfate^[17] as well as antibodies inhibiting cellular binding of envelope glycoproteins^[18]. HCV-like particles (HCV-LP) generated by self-assembly of the HCV structural proteins in insect cells have been shown to exhibit morphologic, biophysical, and antigenic properties similar to putative virions isolated from HCV-infected patients^[10]. In contrast to individually expressed envelope glycoproteins E1 and E2, E1/E2 heterodimers of HCV-LPs are presumably presented in a native, virion-like conformation. HCV-LPs have been shown to bind and enter human hepatoma cells as well as primary hepatocytes and dendritic cells in a receptor-mediated manner, therefore representing a useful model system for the study of HCV-host cell interaction including the characterization of antibodies interfering with cellular binding of particles^[17,19-25].

Retroviral HCV pseudotyped particles (HCVpp) represent a convenient and elegant approach to study

viral entry and antibody-mediated neutralization^[11,12]. Infectious HCVpp consist of unmodified HCV envelope glycoproteins E1 and E2 assembled onto retroviral or lentiviral core particles^[11,12]. HCVpp are produced by transfecting cells with expression vectors encoding the full-length E1/E2 polypeptide, retroviral or lentiviral core proteins, and a packaging-competent retro- or lentiviral genome carrying a marker gene. The presence of a green fluorescent protein or luciferase reporter gene packaged within these HCVpp allows reliable and fast determination of infectivity mediated by the envelope glycoproteins. HCVpp are infectious for certain cell lines of hepatocyte origin, principally Huh-7 cells, as well as for human primary hepatocytes^[11,12]. This system has been extremely useful in identifying neutralizing antibodies as well as characterization of the molecular mechanisms of antibody-mediated neutralization^[12,25-30]. Vesicular stomatitis viruses (VSV)/HCV pseudotypes expressing HCV E1 or E2 chimeric proteins containing transmembrane and cytoplasmic domains of the VSV G glycoprotein have been developed as another HCV pseudotype model system to study HCV entry and antibody-mediated neutralization^[13,31]. VSV/HCV pseudotypes infect human hepatoma cell lines and sera from HCV-infected chimpanzees or humans neutralize the pseudotype virus infectivity^[13,32]. In contrast to retroviral HCV pseudotypes demonstrating strong tropism for liver-derived cell lines, VSV/HCV pseudotypes are generated in relatively lower titer and can infect a broad range of mammalian cell lines, including cell lines not derived from the liver.

Most recently, several laboratories succeeded in establishing the efficient production of infectious HCV particles using a unique clone derived from a viral isolate of a Japanese patient with fulminant hepatitis C (JFH-1)^[14-16]. Successful infection of naïve Huh-7 and Huh-7-derived hepatoma cells with cell-culture derived HCV (HCVcc) was demonstrated by detection of viral proteins and a highly reproducible time-dependent increase of viral RNA in infected cells^[14-16]. Virus production in Huh-7 cells was dependent on an active viral polymerase and expression of a functional viral envelope containing the HCV envelope glycoproteins E1 and E2^[14-16]. Inoculation of naïve chimpanzees with JFH-1 or chimeric J6/JFH-1-derived HCV particles synthesized *in vitro* resulted in viral infection *in vivo* demonstrating the biological significance of this model system^[14,33]. The ability to generate infectious HCVcc of different genotypes – such as the development of chimeric HCVcc or HCVcc derived from HCV genotype prototype 1a strain H77 certainly improves the scope of the cell culture system for HCV infection^[34,35]. Infection of HCVcc has been shown to be efficiently neutralized by anti-HCV antibodies derived from human sera^[14] as well as polyclonal anti-envelope antibodies^[34].

The chimpanzee remains the only natural occurring animal model for the study of HCV infection *in vivo*. The clinical course of infection is usually milder in chimpanzees than in humans. However, these animals have provided unique opportunities to study adaptive immune responses to HCV^[36]. Using the chimpanzee model, antibodies with neutralizing properties have first been described^[37,38]. These antibodies were directed against epitopes in the envelope

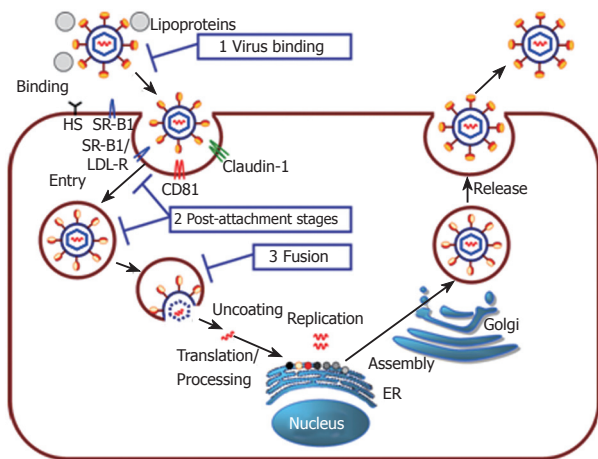


Figure 1 Potential targets for neutralizing antibodies within the HCV life cycle. A model of HCV infection with potential targets for virus neutralizing antibodies is shown. Antibodies can potentially interfere with the viral life cycle at different steps. ER: Endoplasmic reticulum; HS: Heparan sulfate; LDL-R: LDL receptor; SR-BI: Scavenger receptor class B type I .

glycoprotein E2 hypervariable region 1 (HVR-1) of HCV and appeared to be isolate-specific. The chimpanzee model has also been used to study protective immunity against re-exposure. Vaccination studies^[39] and passive immunization with rabbit anti-sera^[38] have shown some protection but infection of chimpanzees with HCV does not provide complete protective immunity against re-infection with homologous or heterologous virus^[40-44].

TARGETS OF HOST NEUTRALIZING RESPONSES

In recent years, rapid progress has been made in the understanding of the molecular mechanisms of HCV life cycle (Figure 1). Attachment of the virus to the target cell is mediated through binding of HCV envelope glycoproteins to binding factors present on the host cell surface, such as the glycosaminoglycan heparan sulfate^[17,25]. Binding and entry of HCV is believed to be a multistep process involving several attachment and entry factors, such as CD81, SR-BI and claudin-1^[45-47]. HCV is most certainly internalized in a clathrin-dependent manner and HCV genome delivery into the host cell cytosol prior to HCV replication is pH dependent^[48-51]. In analogy to other viral infections, antibodies neutralizing HCV may render virions non-infectious by interfering with different steps of the viral life cycle^[52,53]. Binding of the antibodies to the virus may directly block attachment of the virus with the host cell and thus inhibit dissemination of infection. Neutralizing antibodies may also interfere with post-binding steps such as interaction of the virus with host entry factors. If endocytosis is an obligate replicative step, internalization of the virus into the host cell by endocytosis may also cause neutralization. Neutralization of viruses by antibodies may also take place during fusion at the cell surface or in endosomes: Neutralizing antibodies may directly interfere with the fusogenic protein, hinder conformational changes necessary for the fusion process or simply obstruct contact between cellular

Table 1 HCV epitopes targeted by antibodies interfering with cellular HCV envelope glycoprotein binding, viral entry or infection			
Envelope glycoprotein	Epitope (Amino acids)	Potential function	Model system
E1	192-226	Membrane fusion	HCV-LP binding ^[85]
	197-207		HCV-LP binding ^[24]
	270-284		HCVpp entry ^[63]
	313-332		HCV-LP binding ^[85]
E2	HVR-1	SR-BI/heparan sulfate binding	Chimpanzee ^[38]
	HVR-1		E2 binding, HCVpp entry ^[18,27]
	396-407		HCVpp entry ^[12]
	408-422		HCV-LP binding ^[22]
	412-419	Membrane fusion	HCVpp entry ^[55]
	412-423		HCVpp entry, HCVcc infection ^[12,57]
	416-430		HCVpp entry ^[63]
	432-443		HCVpp entry ^[12]
	436-447	CD81 binding	HCVpp entry ^[12]
	474-494		E2 binding, HCVpp entry ^[56,86]
	522-551	CD81 binding	E2 binding, HCVpp entry ^[56,86]
	600-620		HCVpp entry ^[63]
	640-653		HCV-LP binding ^[24]
	644-655		HCVpp entry ^[12]
	CD	Membrane fusion	E2 binding, HCVpp entry ^[58-61]

Envelope glycoprotein epitope (position amino acid within the HCV polypeptide) and its potential function with the viral entry process is shown. The experimental system for antibody-mediated inhibition of viral binding, entry or infection is listed together with the respective reference. CD: Conformation-dependent; E1 and E2: Envelope glycoproteins E1 and E2; HCV-LP: HCV-like particles; HVR-1: Hypervariable region-1; HCVpp: HCV pseudotyped particles; HCVcc: Cell-culture derived HCV; SR-BI: Scavenger receptor class B type I .

and viral membranes. In addition, neutralizing antibodies may also interfere with viral uncoating or the first steps necessary for viral replication (Figure 1). The identification and characterization of antibodies targeting distinct steps of viral entry is thus an important strategy for the understanding of the molecular mechanisms of antibody-mediated neutralization.

Using the above described model systems, it could be demonstrated that envelope glycoproteins E1 and E2 are critical for host cell entry and thus represent important targets for virus neutralization. Monoclonal or polyclonal antibodies targeting both linear and conformational epitopes of envelope glycoprotein E2 have been shown to inhibit cellular binding of HCV-LP binding, entry of HCVpp and infection of HCVcc (Table 1)^[10-12,14-16,19,20]. Several viral epitopes targeted by neutralizing antibodies have already been identified: epitopes of the E2 HVR-1 region (aa 384-410)^[12,18,27], two epitopes adjacent to the N-terminal HVR-1 region (aa 408-422 and aa 412-419)^[22,54,55], the E2 CD81 binding region (aa 474-494 and aa 522-551)^[12,54,56,57] and conformational epitopes within glycoprotein E2^[58-61]. These epitopes may represent potential candidate targets for antibodies in passive immunoprophylaxis. Indeed, two studies have demonstrated that monoclonal antibodies directed against

conformational epitopes^[60] or epitope aa 412-423 exhibited broad cross-neutralizing activity among all major genotypes of HCVpp entry^[54] as well as HCVcc infectivity^[62]. Most recently, at least three epitopes (aa 270-284, 416-430, 600-620) playing a role in membrane fusion processes have been identified in the envelope glycoproteins E1 and E2^[63]. Since one epitope (aa 416-430) has been shown to represent a target for monoclonal antibodies efficiently neutralizing HCV infection^[54], it is conceivable that membrane fusion may represent another target for anti-HCV antibodies with neutralizing properties.

IMPACT OF VIRUS NEUTRALIZING ANTIBODIES FOR PATHOGENESIS OF INFECTION

HCV RNA is detectable already one week following infection. Despite the rapid onset of viral replication, there is a delay in the appearance of HCV-specific T-lymphocytes and HCV-specific antibodies which only appear several weeks after infection. Patients who spontaneously clear HCV infection have been described to mount a vigorous multi-epitope-specific CD4 and CD8 T-cell response^[64,65]. Antibody-mediated neutralization occurs during HCV infection *in vivo* but the role of antibodies for the control of HCV infection has been difficult to study. Antibody-mediated neutralization has been suggested by study of patients undergoing liver transplantation for HCV- and hepatitis B virus (HBV)-related liver cirrhosis. Infusion of anti-HBs hyperimmune globulin containing anti-HCV appeared to reduce HCV infection in the transplanted liver^[66]. In addition, HCV-infected patients with primary antibody deficiencies have been reported to have accelerated rates of disease progression^[67,68]. Moreover, passive protection against HCV has been demonstrated in a cohort of patients that had been administered immunoglobulin preparations derived from HCV RNA-positive plasma but containing HCV-neutralizing antibodies^[69]. However, in the majority of patients, HCV infection is established despite the induction of an humoral immune response that targets various epitopes of the HCV envelope glycoproteins^[22,26,28,70,71].

Until recently, functional studies analyzing the neutralizing antibody response during acute and chronic HCV infection using HCV model systems demonstrated a lack of neutralizing antibodies in the majority of patients with acute HCV infection^[22,26,70,72]. These studies were limited by the fact that the viral surrogate ligand was derived from a different isolate than the virus present in the infected patient thus precluding the detection of isolate-specific antibodies. Most recently, studies using well defined nosocomial or single-source HCV outbreaks with a defined inoculum enabled to study the role of isolate-specific neutralizing antibodies for control of HCV infection in humans. Using the HCVpp model system, two studies have demonstrated that neutralizing antibodies are induced in the early phase of infection by patients who subsequently clear the virus^[29] or control viral infection^[73]. In a well characterized single-source outbreak of hepatitis C, viral clearance was associated with

a rapid induction of neutralizing antibodies in the early phase of infection. In contrast, chronic HCV infection was characterized by absent or low-titer neutralizing antibodies in the early phase of infection^[29]. In addition, patients with resolution of infection were shown to exhibit a broader cross-neutralizing activity of antibodies in the early phase of infection. An impaired ability to cross-neutralize viral variants rapidly emerging during acute infection may thus contribute to viral evasion from neutralizing responses in persistent HCV infection^[29]. These results suggest that a strong early broad neutralizing antibody response may contribute to control of HCV in the acute phase of infection and assist cellular immune responses in viral clearance. This conclusion is further supported by recent findings for HIV demonstrating that neutralizing antibodies act in concert with antiviral cellular responses for control of HIV infection^[74-76]. Furthermore, experimental data obtained in animal models have demonstrated that immune control of poorly cytopathic viruses, such as lymphocytic choriomeningitis virus (LCMV) or simian immunodeficiency virus requires a collaboration of both the cellular and humoral arms of the immune system^[77]. Indeed, gene-targeted mice expressing the immunoglobulin heavy chain of virus-neutralizing antibodies exhibit an accelerated LCMV clearance^[75]. Applying these findings to HCV infection- another prototype of persistent-prone non-cytopathic viruses- it is conceivable, that both cellular^[5,64,65,78,79] and neutralizing responses^[29,73] may contribute to control of HCV infection during the very early phase of viral infection.

Patients who do not clear the virus develop high-titer and even cross-neutralizing antibodies during the chronic phase of infection^[14,22,26,29,70,80]. Paradoxically, these antibodies are not able to control HCV infection. Viral escape from antibody-mediated neutralization in these patients may occur on several levels: (1) HCV exists as a quasispecies with distinct viral variants in infected individuals changing constantly over time and his variability has been shown to represent a mechanism of escape from antibody-mediated neutralization in the chimpanzee model^[26]; (2) the interplay of HCV glycoproteins with high-density lipoprotein and SR-BI has been shown to mediate protection from neutralizing antibodies present in sera of acute and chronic HCV-infected patients^[27,74]; (3) as shown for other viruses such as human immunodeficiency virus (HIV), escape from neutralizing antibodies may occur through a combination of different mechanisms, for instance point mutations, insertions/deletions or changes in glycosylation patterns of the viral envelope^[30,81] or conformational masking of receptor binding sites following envelope-antibody interaction^[82] preventing neutralizing antibody binding^[83].

Most recently, it has been shown for a chronic HCV patient who has been meticulously followed-up for 30 years that HCV continuously escapes the host's immune system by repeated mutational changes resulting in loss of recognition of the HCV envelope glycoproteins by antibodies^[80]. In fact, neutralization of heterologous strains does not reflect neutralization of the viral variants present in the patient's serum at the time of sampling^[80]. These data suggest that the neutralizing antibody response of

the host lags behind the rapidly evolving HCV envelope glycoprotein sequences of the quasispecies population. The fact that envelope glycoprotein sequences and neutralizing antibody specificity change over time suggest that neutralizing antibodies exert selective pressure on HCV evolution. In line with this hypothesis, it has been shown that HCV quasispecies complexity is associated with the inability to clear HCV infection and development of chronic disease^[84].

CONCLUSION

The development of robust tissue culture model systems for HCV infection within recent years has finally allowed for the study of antibody-mediated neutralization in HCV infection. Rapid progress has since then been made in determining the kinetic and targets of host neutralizing responses in the course of HCV infection. The novel model systems and patient cohorts with well defined viral isolates will now allow the identification of the molecular mechanisms of antibody-mediated neutralization as well as mechanisms of viral escape from host neutralizing responses. The elucidation of these mechanisms will be crucial for the understanding of HCV pathogenesis as well as the development of novel preventive and therapeutic strategies for control of HCV infection.

REFERENCES

- 1 **Chisari FV**. Unscrambling hepatitis C virus-host interactions. *Nature* 2005; **436**: 930-932
- 2 **Lindenbach BD**, Thiel HJ and Rice CM. Flaviviridae: the viruses and their replication. In: Knipe DM and Howley PM. *Fields Virology*. Philadelphia: Lippincott-Raven, 2007: 1101-1152
- 3 **Sung VM**, Shimodaira S, Doughty AL, Picchio GR, Can H, Yen TS, Lindsay KL, Levine AM, Lai MM. Establishment of B-cell lymphoma cell lines persistently infected with hepatitis C virus in vivo and in vitro: the apoptotic effects of virus infection. *J Virol* 2003; **77**: 2134-2146
- 4 **Goutagny N**, Fatmi A, De Ledinghen V, Penin F, Couzigou P, Inchauspé G, Bain C. Evidence of viral replication in circulating dendritic cells during hepatitis C virus infection. *J Infect Dis* 2003; **187**: 1951-1958
- 5 **Rehermann B**, Nascimbeni M. Immunology of hepatitis B virus and hepatitis C virus infection. *Nat Rev Immunol* 2005; **5**: 215-229
- 6 **Parren PW**, Burton DR. The antiviral activity of antibodies in vitro and in vivo. *Adv Immunol* 2001; **77**: 195-262
- 7 **Hangartner L**, Zinkernagel RM, Hangartner H. Antiviral antibody responses: the two extremes of a wide spectrum. *Nat Rev Immunol* 2006; **6**: 231-243
- 8 **Pileri P**, Uematsu Y, Campagnoli S, Galli G, Falugi F, Petracca R, Weiner AJ, Houghton M, Rosa D, Grandi G, Abrignani S. Binding of hepatitis C virus to CD81. *Science* 1998; **282**: 938-941
- 9 **Scarselli E**, Ansuini H, Cerino R, Roccasecca RM, Acali S, Filocamo G, Traboni C, Nicosia A, Cortese R, Vitelli A. The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. *EMBO J* 2002; **21**: 5017-5025
- 10 **Baumert TF**, Ito S, Wong DT, Liang TJ. Hepatitis C virus structural proteins assemble into viruslike particles in insect cells. *J Virol* 1998; **72**: 3827-3836
- 11 **Bartosch B**, Dubuisson J, Cosset FL. Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complexes. *J Exp Med* 2003; **197**: 633-642
- 12 **Hsu M**, Zhang J, Flint M, Logvinoff C, Cheng-Mayer C, Rice CM, McKeating JA. Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles. *Proc Natl Acad Sci USA* 2003; **100**: 7271-7276
- 13 **Lagging LM**, Meyer K, Owens RJ, Ray R. Functional role of hepatitis C virus chimeric glycoproteins in the infectivity of pseudotyped virus. *J Virol* 1998; **72**: 3539-3546
- 14 **Wakita T**, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, Murthy K, Habermann A, Kräusslich HG, Mizokami M, Bartenschlager R, Liang TJ. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* 2005; **11**: 791-796
- 15 **Lindenbach BD**, Evans MJ, Syder AJ, Wölk B, Tellinghuisen TL, Liu CC, Maruyama T, Hynes RO, Burton DR, McKeating JA, Rice CM. Complete replication of hepatitis C virus in cell culture. *Science* 2005; **309**: 623-626
- 16 **Zhong J**, Gastaminza P, Cheng G, Kapadia S, Kato T, Burton DR, Wieland SF, Uprichard SL, Wakita T, Chisari FV. Robust hepatitis C virus infection in vitro. *Proc Natl Acad Sci USA* 2005; **102**: 9294-9299
- 17 **Barth H**, Schafer C, Adah MI, Zhang F, Linhardt RJ, Toyoda H, Kinoshita-Toyoda A, Toida T, Van Kuppevelt TH, Depla E, Von Weizsäcker F, Blum HE, Baumert TF. Cellular binding of hepatitis C virus envelope glycoprotein E2 requires cell surface heparan sulfate. *J Biol Chem* 2003; **278**: 41003-41012
- 18 **Rosa D**, Campagnoli S, Moretto C, Guenzi E, Cousens L, Chin M, Dong C, Weiner AJ, Lau JY, Choo QL, Chien D, Pileri P, Houghton M, Abrignani S. A quantitative test to estimate neutralizing antibodies to the hepatitis C virus: cytofluorimetric assessment of envelope glycoprotein 2 binding to target cells. *Proc Natl Acad Sci USA* 1996; **93**: 1759-1763
- 19 **Barth H**, Cerino R, Arcuri M, Hoffmann M, Schürmann P, Adah MI, Gissler B, Zhao X, Ghisetti V, Lavezzo B, Blum HE, von Weizsäcker F, Vitelli A, Scarselli E, Baumert TF. Scavenger receptor class B type I and hepatitis C virus infection of primary tupaia hepatocytes. *J Virol* 2005; **79**: 5774-5785
- 20 **Wellnitz S**, Klumpp B, Barth H, Ito S, Depla E, Dubuisson J, Blum HE, Baumert TF. Binding of hepatitis C virus-like particles derived from infectious clone H77C to defined human cell lines. *J Virol* 2002; **76**: 1181-1193
- 21 **Barth H**, Ulsenheimer A, Pape GR, Diepolder HM, Hoffmann M, Neumann-Haefelin C, Thimme R, Henneke P, Klein R, Paranhos-Baccalà G, Depla E, Liang TJ, Blum HE, Baumert TF. Uptake and presentation of hepatitis C virus-like particles by human dendritic cells. *Blood* 2005; **105**: 3605-3614
- 22 **Steinmann D**, Barth H, Gissler B, Schürmann P, Adah MI, Gerlach JT, Pape GR, Depla E, Jacobs D, Maertens G, Patel AH, Inchauspé G, Liang TJ, Blum HE, Baumert TF. Inhibition of hepatitis C virus-like particle binding to target cells by antiviral antibodies in acute and chronic hepatitis C. *J Virol* 2004; **78**: 9030-9040
- 23 **Triyatni M**, Saunier B, Maruvada P, Davis AR, Ulianich L, Heller T, Patel A, Kohn LD, Liang TJ. Interaction of hepatitis C virus-like particles and cells: a model system for studying viral binding and entry. *J Virol* 2002; **76**: 9335-9344
- 24 **Triyatni M**, Vergalla J, Davis AR, Hadlock KG, Fount SK, Liang TJ. Structural features of envelope proteins on hepatitis C virus-like particles as determined by anti-envelope monoclonal antibodies and CD81 binding. *Virology* 2002; **298**: 124-132
- 25 **Barth H**, Schnober EK, Zhang F, Linhardt RJ, Depla E, Boson B, Cosset FL, Patel AH, Blum HE, Baumert TF. Viral and cellular determinants of the hepatitis C virus envelope-heparan sulfate interaction. *J Virol* 2006; **80**: 10579-10590
- 26 **Bartosch B**, Bukh J, Meunier JC, Granier C, Engle RE, Blackwelder WC, Emerson SU, Cosset FL, Purcell RH. In vitro assay for neutralizing antibody to hepatitis C virus: evidence for broadly conserved neutralization epitopes. *Proc Natl Acad Sci USA* 2003; **100**: 14199-14204
- 27 **Bartosch B**, Verney G, Dreux M, Donot P, Morice Y, Penin F, Pawlotsky JM, Lavillette D, Cosset FL. An interplay between hypervariable region 1 of the hepatitis C virus E2 glycoprotein, the scavenger receptor BI, and high-density lipoprotein promotes both enhancement of infection and protection against neutralizing antibodies. *J Virol* 2005; **79**: 8217-8229
- 28 **Meunier JC**, Engle RE, Faulk K, Zhao M, Bartosch B, Alter

- H, Emerson SU, Cosset FL, Purcell RH, Bukh J. Evidence for cross-genotype neutralization of hepatitis C virus pseudoparticles and enhancement of infectivity by apolipoprotein C1. *Proc Natl Acad Sci USA* 2005; **102**: 4560-4565
- 29 **Pestka JM**, Zeisel MB, Bläser E, Schürmann P, Bartosch B, Cosset FL, Patel AH, Meisel H, Baumert J, Viazov S, Rispeter K, Blum HE, Roggendorf M, Baumert TF. Rapid induction of virus-neutralizing antibodies and viral clearance in a single-source outbreak of hepatitis C. *Proc Natl Acad Sci USA* 2007; **104**: 6025-6030
 - 30 **Helle F**, Goffard A, Morel V, Duverlie G, McKeating J, Keck ZY, Fong S, Penin F, Dubuisson J, Voisset C. The neutralizing activity of anti-hepatitis C virus antibodies is modulated by specific glycans on the E2 envelope protein. *J Virol* 2007; **81**: 8101-8111
 - 31 **Buonocore L**, Blight KJ, Rice CM, Rose JK. Characterization of vesicular stomatitis virus recombinants that express and incorporate high levels of hepatitis C virus glycoproteins. *J Virol* 2002; **76**: 6865-6872
 - 32 **Meyer K**, Beyene A, Bowlin TL, Basu A, Ray R. Coexpression of hepatitis C virus E1 and E2 chimeric envelope glycoproteins displays separable ligand sensitivity and increases pseudotype infectious titer. *J Virol* 2004; **78**: 12838-12847
 - 33 **Lindenbach BD**, Meuleman P, Ploss A, Vanwolleghem T, Syder AJ, McKeating JA, Lanford RE, Feinstone SM, Major ME, Leroux-Roels G, Rice CM. Cell culture-grown hepatitis C virus is infectious in vivo and can be recultured in vitro. *Proc Natl Acad Sci USA* 2006; **103**: 3805-3809
 - 34 **Pietschmann T**, Kaul A, Koutsoudakis G, Shavinskaya A, Kallis S, Steinmann E, Abid K, Negro F, Dreux M, Cosset FL, Bartenschlager R. Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras. *Proc Natl Acad Sci USA* 2006; **103**: 7408-7413
 - 35 **Yi M**, Villanueva RA, Thomas DL, Wakita T, Lemon SM. Production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells. *Proc Natl Acad Sci USA* 2006; **103**: 2310-2315
 - 36 **Bukh J**. A critical role for the chimpanzee model in the study of hepatitis C. *Hepatology* 2004; **39**: 1469-1475
 - 37 **Farci P**, Alter HJ, Wong DC, Miller RH, Govindarajan S, Engle R, Shapiro M, Purcell RH. Prevention of hepatitis C virus infection in chimpanzees after antibody-mediated in vitro neutralization. *Proc Natl Acad Sci USA* 1994; **91**: 7792-7796
 - 38 **Farci P**, Shimoda A, Wong D, Cabezon T, De Gioannis D, Strazzer A, Shimizu Y, Shapiro M, Alter HJ, Purcell RH. Prevention of hepatitis C virus infection in chimpanzees by hyperimmune serum against the hypervariable region 1 of the envelope 2 protein. *Proc Natl Acad Sci USA* 1996; **93**: 15394-15399
 - 39 **Choo QL**, Kuo G, Ralston R, Weiner A, Chien D, Van Nest G, Han J, Berger K, Thudium K, Kuo C. Vaccination of chimpanzees against infection by the hepatitis C virus. *Proc Natl Acad Sci USA* 1994; **91**: 1294-1298
 - 40 **Farci P**, Alter HJ, Govindarajan S, Wong DC, Engle R, Lesniewski RR, Mushahwar IK, Desai SM, Miller RH, Ogata N. Lack of protective immunity against reinfection with hepatitis C virus. *Science* 1992; **258**: 135-140
 - 41 **Prince AM**, Brotman B, Huima T, Pascual D, Jaffery M, Inchauspé G. Immunity in hepatitis C infection. *J Infect Dis* 1992; **165**: 438-443
 - 42 **Bassett SE**, Guerra B, Brasky K, Miskovsky E, Houghton M, Klimpel GR, Lanford RE. Protective immune response to hepatitis C virus in chimpanzees rechallenged following clearance of primary infection. *Hepatology* 2001; **33**: 1479-1487
 - 43 **Major ME**, Mihalik K, Puig M, Rehmann B, Nascimbeni M, Rice CM, Feinstone SM. Previously infected and recovered chimpanzees exhibit rapid responses that control hepatitis C virus replication upon rechallenge. *J Virol* 2002; **76**: 6586-6595
 - 44 **Lanford RE**, Guerra B, Chavez D, Bigger C, Brasky KM, Wang XH, Ray SC, Thomas DL. Cross-genotype immunity to hepatitis C virus. *J Virol* 2004; **78**: 1575-1581
 - 45 **Koutsoudakis G**, Kaul A, Steinmann E, Kallis S, Lohmann V, Pietschmann T, Bartenschlager R. Characterization of the early steps of hepatitis C virus infection by using luciferase reporter viruses. *J Virol* 2006; **80**: 5308-5320
 - 46 **Zeisel MB**, Koutsoudakis G, Schnober EK, Haberstroth A, Blum HE, Cosset FL, Wakita T; Jaeck D, Doffoel M, Royer C, Soulier E, Schvoerer E, Schuster C, Stoll-Keller F, Bartenschlager R, Pietschmann T, Barth H, Baumert TF. Scavenger receptor BI is a key host factor for Hepatitis C virus infection required for an entry step closely linked to CD81. *Hepatology* 2007; in press
 - 47 **Evans MJ**, von Hahn T, Tscherne DM, Syder AJ, Panis M, Wölk B, Hatzioannou T, McKeating JA, Bieniasz PD, Rice CM. Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. *Nature* 2007; **446**: 801-805
 - 48 **Blanchard E**, Belouzard S, Goueslain L, Wakita T, Dubuisson J, Wychowski C, Rouillé Y. Hepatitis C virus entry depends on clathrin-mediated endocytosis. *J Virol* 2006; **80**: 6964-6972
 - 49 **Meertens L**, Bertaux C, Dragic T. Hepatitis C virus entry requires a critical postinternalization step and delivery to early endosomes via clathrin-coated vesicles. *J Virol* 2006; **80**: 11571-11578
 - 50 **Codran A**, Royer C, Jaeck D, Bastien-Valle M, Baumert TF, Kieny MP, Pereira CA, Martin JP. Entry of hepatitis C virus pseudotypes into primary human hepatocytes by clathrin-dependent endocytosis. *J Gen Virol* 2006; **87**: 2583-2593
 - 51 **Tscherne DM**, Jones CT, Evans MJ, Lindenbach BD, McKeating JA, Rice CM. Time- and temperature-dependent activation of hepatitis C virus for low-pH-triggered entry. *J Virol* 2006; **80**: 1734-1741
 - 52 **Barth H**, Liang TJ, Baumert TF. Hepatitis C virus entry: molecular biology and clinical implications. *Hepatology* 2006; **44**: 527-535
 - 53 **Klasse PJ**, Sattentau QJ. Occupancy and mechanism in antibody-mediated neutralization of animal viruses. *J Gen Virol* 2002; **83**: 2091-2108
 - 54 **Owsianka A**, Tarr AW, Juttla VS, Lavillette D, Bartosch B, Cosset FL, Ball JK, Patel AH. Monoclonal antibody AP33 defines a broadly neutralizing epitope on the hepatitis C virus E2 envelope glycoprotein. *J Virol* 2005; **79**: 11095-11104
 - 55 **Zhang P**, Wu CG, Mihalik K, Virata-Theimer ML, Yu MY, Alter HJ, Feinstone SM. Hepatitis C virus epitope-specific neutralizing antibodies in Igs prepared from human plasma. *Proc Natl Acad Sci USA* 2007; **104**: 8449-8454
 - 56 **Zhang J**, Randall G, Higginbottom A, Monk P, Rice CM, McKeating JA. CD81 is required for hepatitis C virus glycoprotein-mediated viral infection. *J Virol* 2004; **78**: 1448-1455
 - 57 **Owsianka AM**, Timms JM, Tarr AW, Brown RJ, Hickling TP, Szwejk A, Bienkowska-Szewczyk K, Thomson BJ, Patel AH, Ball JK. Identification of conserved residues in the E2 envelope glycoprotein of the hepatitis C virus that are critical for CD81 binding. *J Virol* 2006; **80**: 8695-8704
 - 58 **Habersetzer F**, Fournillier A, Dubuisson J, Rosa D, Abrignani S, Wychowski C, Nakano I, Trépo C, Desgranges C, Inchauspé G. Characterization of human monoclonal antibodies specific to the hepatitis C virus glycoprotein E2 with in vitro binding neutralization properties. *Virology* 1998; **249**: 32-41
 - 59 **Keck ZY**, Op De Beeck A, Hadlock KG, Xia J, Li TK, Dubuisson J, Fong SK. Hepatitis C virus E2 has three immunogenic domains containing conformational epitopes with distinct properties and biological functions. *J Virol* 2004; **78**: 9224-9232
 - 60 **Schofield DJ**, Bartosch B, Shimizu YK, Allander T, Alter HJ, Emerson SU, Cosset FL, Purcell RH. Human monoclonal antibodies that react with the E2 glycoprotein of hepatitis C virus and possess neutralizing activity. *Hepatology* 2005; **42**: 1055-1062
 - 61 **Op De Beeck A**, Voisset C, Bartosch B, Ciczora Y, Cocquerel L, Keck Z, Fong S, Cosset FL, Dubuisson J. Characterization of functional hepatitis C virus envelope glycoproteins. *J Virol* 2004; **78**: 2994-3002
 - 62 **Tarr AW**, Owsianka AM, Timms JM, McClure CP, Brown RJ, Hickling TP, Pietschmann T, Bartenschlager R, Patel AH, Ball JK. Characterization of the hepatitis C virus E2 epitope defined by the broadly neutralizing monoclonal antibody

- AP33. *Hepatology* 2006; **43**: 592-601
- 63 **Lavillette D**, Pécheur EI, Donot P, Fresquet J, Molle J, Corbau R, Dreux M, Penin F, Cosset FL. Characterization of fusion determinants points to the involvement of three discrete regions of both e1 and e2 glycoproteins in the membrane fusion process of hepatitis C virus. *J Virol* 2007; **81**: 8752-8765
 - 64 **Diepolder HM**, Zachoval R, Hoffmann RM, Wierenga EA, Santantonio T, Jung MC, Eichenlaub D, Pape GR. Possible mechanism involving T-lymphocyte response to non-structural protein 3 in viral clearance in acute hepatitis C virus infection. *Lancet* 1995; **346**: 1006-1007
 - 65 **Thimme R**, Oldach D, Chang KM, Steiger C, Ray SC, Chisari FV. Determinants of viral clearance and persistence during acute hepatitis C virus infection. *J Exp Med* 2001; **194**: 1395-1406
 - 66 **Féray C**, Gigou M, Samuel D, Ducot B, Maisonneuve P, Reynès M, Bismuth A, Bismuth H. Incidence of hepatitis C in patients receiving different preparations of hepatitis B immunoglobulins after liver transplantation. *Ann Intern Med* 1998; **128**: 810-816
 - 67 **Chapel HM**, Christie JM, Peach V, Chapman RW. Five-year follow-up of patients with primary antibody deficiencies following an outbreak of acute hepatitis C. *Clin Immunol* 2001; **99**: 320-324
 - 68 **Christie JM**, Healey CJ, Watson J, Wong VS, Duddridge M, Snowden N, Rosenberg WM, Fleming KA, Chapel H, Chapman RW. Clinical outcome of hypogammaglobulinaemic patients following outbreak of acute hepatitis C: 2 year follow up. *Clin Exp Immunol* 1997; **110**: 4-8
 - 69 **Yu MY**, Bartosch B, Zhang P, Guo ZP, Renzi PM, Shen LM, Granier C, Feinstone SM, Cosset FL, Purcell RH. Neutralizing antibodies to hepatitis C virus (HCV) in immune globulins derived from anti-HCV-positive plasma. *Proc Natl Acad Sci USA* 2004; **101**: 7705-7710
 - 70 **Logvinoff C**, Major ME, Oldach D, Heyward S, Talal A, Balfe P, Feinstone SM, Alter H, Rice CM, McKeating JA. Neutralizing antibody response during acute and chronic hepatitis C virus infection. *Proc Natl Acad Sci USA* 2004; **101**: 10149-10154
 - 71 **Hadlock KG**, Gish R, Rowe J, Rajyaguru SS, Newsom M, Warford A, Fong SK. Cross-reactivity and clinical impact of the antibody response to hepatitis C virus second envelope glycoprotein (E2). *J Med Virol* 2001; **65**: 23-29
 - 72 **Netski DM**, Mosbruger T, Depla E, Maertens G, Ray SC, Hamilton RG, Roundtree S, Thomas DL, McKeating J, Cox A. Humoral immune response in acute hepatitis C virus infection. *Clin Infect Dis* 2005; **41**: 667-675
 - 73 **Lavillette D**, Morice Y, Germanidis G, Donot P, Soulier A, Pagkalos E, Sakellariou G, Intrator L, Bartosch B, Pawlotsky JM, Cosset FL. Human serum facilitates hepatitis C virus infection, and neutralizing responses inversely correlate with viral replication kinetics at the acute phase of hepatitis C virus infection. *J Virol* 2005; **79**: 6023-6034
 - 74 **Dreux M**, Pietschmann T, Granier C, Voisset C, Ricard-Blum S, Mangeot PE, Keck Z, Fong S, Vu-Dac N, Dubuisson J, Bartenschlager R, Lavillette D, Cosset FL. High density lipoprotein inhibits hepatitis C virus-neutralizing antibodies by stimulating cell entry via activation of the scavenger receptor BI. *J Biol Chem* 2006; **281**: 18285-18295
 - 75 **Hangartner L**, Senn BM, Ledermann B, Kalinke U, Seiler P, Bucher E, Zellweger RM, Fink K, Odermatt B, Bürki K, Zinkernagel RM, Hangartner H. Antiviral immune responses in gene-targeted mice expressing the immunoglobulin heavy chain of virus-neutralizing antibodies. *Proc Natl Acad Sci USA* 2003; **100**: 12883-12888
 - 76 **Igarashi T**, Brown C, Azadegan A, Haigwood N, Dimitrov D, Martin MA, Shibata R. Human immunodeficiency virus type 1 neutralizing antibodies accelerate clearance of cell-free virions from blood plasma. *Nat Med* 1999; **5**: 211-216
 - 77 **Ciurea A**, Klenerman P, Hunziker L, Horvath E, Senn BM, Ochsenbein AF, Hangartner H, Zinkernagel RM. Viral persistence in vivo through selection of neutralizing antibody-escape variants. *Proc Natl Acad Sci USA* 2000; **97**: 2749-2754
 - 78 **Takaki A**, Wiese M, Maertens G, Depla E, Seifert U, Liebetrau A, Miller JL, Manns MP, Rehermann B. Cellular immune responses persist and humoral responses decrease two decades after recovery from a single-source outbreak of hepatitis C. *Nat Med* 2000; **6**: 578-582
 - 79 **Bowen DG**, Walker CM. Adaptive immune responses in acute and chronic hepatitis C virus infection. *Nature* 2005; **436**: 946-952
 - 80 **von Hahn T**, Yoon JC, Alter H, Rice CM, Rehermann B, Balfe P, McKeating JA. Hepatitis C virus continuously escapes from neutralizing antibody and T-cell responses during chronic infection in vivo. *Gastroenterology* 2007; **132**: 667-678
 - 81 **Wei X**, Decker JM, Wang S, Hui H, Kappes JC, Wu X, Salazar-Gonzalez JF, Salazar MG, Kilby JM, Saag MS, Komarova NL, Nowak MA, Hahn BH, Kwong PD, Shaw GM. Antibody neutralization and escape by HIV-1. *Nature* 2003; **422**: 307-312
 - 82 **Kwong PD**, Doyle ML, Casper DJ, Cicala C, Leavitt SA, Majeed S, Steenbeke TD, Venturi M, Chaiken I, Fung M, Katinger H, Parren PW, Robinson J, Van Ryk D, Wang L, Burton DR, Freire E, Wyatt R, Sodroski J, Hendrickson WA, Arthos J. HIV-1 evades antibody-mediated neutralization through conformational masking of receptor-binding sites. *Nature* 2002; **420**: 678-682
 - 83 **Srivastava IK**, Ulmer JB, Barnett SW. Role of neutralizing antibodies in protective immunity against HIV. *Hum Vaccin* 2005; **1**: 45-60
 - 84 **Farci P**, Shimoda A, Coiana A, Diaz G, Peddis G, Melpolder JC, Strazzera A, Chien DY, Munoz SJ, Balestrieri A, Purcell RH, Alter HJ. The outcome of acute hepatitis C predicted by the evolution of the viral quasispecies. *Science* 2000; **288**: 339-344
 - 85 **Fournillier A**, Wychowski C, Boucreux D, Baumert TF, Meunier JC, Jacobs D, Muguet S, Depla E, Inchauspé G. Induction of hepatitis C virus E1 envelope protein-specific immune response can be enhanced by mutation of N-glycosylation sites. *J Virol* 2001; **75**: 12088-12097
 - 86 **Flint M**, Maidens C, Loomis-Price LD, Shotton C, Dubuisson J, Monk P, Higginbottom A, Levy S, McKeating JA. Characterization of hepatitis C virus E2 glycoprotein interaction with a putative cellular receptor, CD81. *J Virol* 1999; **73**: 6235-6244

S- Editor Ma N L- Editor Alpini GD E- Editor Yin DH

Robert Thimme, MD, Professor, Series Editor

CD4+ T cell responses in hepatitis C virus infection

Nasser Semmo, Paul Klenerman

Nasser Semmo, Department of Medicine II, University Hospital Freiburg, Hugstetter Strasse 55, 79106 Freiburg, Germany
Paul Klenerman, Nuffield Department of Clinical Medicine, University of Oxford, The Peter Medawar Building for Pathogen Research, South Parks Road, Oxford OX1 3SY, United Kingdom
Supported by the Deutsche Forschungsgemeinschaft and the Wellcome Trust and the James Martin School for the 21st century, Oxford

Correspondence to: Paul Klenerman, Nuffield Department of Clinical Medicine, University of Oxford, The Peter Medawar Building for Pathogen Research, South Parks Road, Oxford OX1 3SY, United Kingdom. paul.klenerman@medawar.ox.ac.uk
Telephone: +44-186-5281885 Fax: +44-186-5281236
Received: June 26, 2007 Revised: July 9, 2007

Abstract

Hepatitis C virus (HCV) infection is a major cause of liver damage, with virus-induced end-stage disease such as liver cirrhosis and hepatocellular carcinoma resulting in a high rate of morbidity and mortality worldwide. Evidence that CD4+ T cell responses to HCV play an important role in the outcome of acute infection has been shown in several studies. However, the mechanisms behind viral persistence and the failure of CD4+ T cell responses to contain virus are poorly understood. During chronic HCV infection, HCV-specific CD4+ T cell responses are relatively weak or absent whereas in resolved infection these responses are vigorous and multispecific. Persons with a T-helper type I profile, which promotes cellular effector mechanisms are thought to be more likely to experience viral clearance, but the overall role of these cells in the immunopathogenesis of chronic liver disease is not known. To define this, much more data is required on the function and specificity of virus-specific CD4+ T cells, especially in the early phases of acute disease and in the liver during chronic infection. The role and possible mechanisms of action of CD4+ T cell responses in determining the outcome of acute and chronic HCV infection will be discussed in this review.

© 2007 WJG. All rights reserved.

Key words: Hepatitis C virus; CD4 T cells; HLA class II; Immune responses; Cytokines; Interleukin 2; Proliferation; Escape; Exhaustion

Semmo N, Klenerman P. CD4+ T cell responses in hepatitis C virus infection. *World J Gastroenterol* 2007; 13(36): 4831-4838

<http://www.wjgnet.com/1007-9327/13/4831.asp>

INTRODUCTION

Hepatitis C virus (HCV) infection is a major cause of liver damage, with virus-induced end-stage disease such as liver cirrhosis and hepatocellular carcinoma resulting in a high rate of morbidity and mortality worldwide. Since the discovery of the virus, considerable evidence has emerged that CD4+ T cell responses to HCV play a key role in the outcome of infection. However many questions remain and these will be discussed in this review.

Cellular immune responses, involving both CD8+ cytotoxic T lymphocytes (CTL) and CD4+ T-helper cells, play an essential role in the control of HCV infection, as they do in other persistent viral diseases. Whereas CTLs are traditionally thought to be the main effector cells that eliminate HCV-infected cells^[1], it is clear that HCV-specific CD4+ T cells also play a critical role. These cells can potentially act in multiple ways and are central to the initiation and maintenance of adaptive immunity. Two likely major roles are in providing help for CD8+ T cells by cytokine production and activation of antigen-presenting cells, but there are multiple other mechanisms of action including direct antiviral effects, a role in B cell maturation, and regulatory functions.

A very clear example of the overall importance of HCV-specific CD4+ T cell responses *in vivo* is an experiment where the antibody-mediated depletion of CD4+ T cells before re-infection of two immune chimpanzees was performed. Such depletion resulted in persistent, low-level viraemia despite functional intra-hepatic memory CD8+ T cell responses^[2]. In this experiment incomplete control of HCV replication by memory CD8+ T cells in the absence of adequate CD4+ T cell help was associated with emergence of viral escape mutations in Class I MHC-restricted epitopes and failure to resolve HCV infection^[2]. This experiment is important in that it shows that CD4+ T cells are necessary for resolution of HCV infection. However, in this case the exact function of the CD4+ T cells was not elucidated. The fact that CD8+ T cell responses were maintained and were functional (inducing viral escape) suggests that their role is not purely in providing support for CD8+ T cell responses.

Overall, most data on CD4+ T cells comes from experiments in infected persons, particularly those

comparing chronic and resolved infection. During chronic HCV infection, HCV-specific CD4⁺ T cell responses are typically described as weak or absent whereas in resolved infection these responses are generally vigorous and multispecific. However, much of the data is derived from cross sectional studies and also based on analysis of one function-proliferation *in vitro*. There is no doubt that CD4⁺ T cell responses in resolved infection and in persistent infection look very different, but how they diverge and what the true functionality of the populations are is not yet clear. In our view, given their central importance in adaptive immunity, understanding why CD4⁺ T cell responses may fail in acute infection is the key question in HCV pathogenesis. Current data on the function and role of virus-specific CD4⁺ T-helper cells in acute and chronic HCV infection will be reviewed here and models discussed.

CD4⁺ T CELL RESPONSES IN ACUTE HCV INFECTION

Clearly, the best place to study the CD4⁺ T cell response against HCV is at the site of infection, but CD4⁺ T cell responses in the liver of acute HCV infection in humans have not been characterized to date. In chimpanzees, liver CD4⁺ T cells have been expanded with anti-CD3 and IL-2 and then tested for proliferation in response to HCV proteins^[3]. In those who failed to control the virus, no CD4⁺ T cell responses were identified whereas in those chimps who transiently or permanently controlled the virus, strong proliferative CD4⁺ T cell responses were detectable. This experiment is limited by the need to expand the cells first *in vitro*, which might exclude certain cell populations, especially at the peak of infection. What is interesting is not only the dichotomy between those animals that cleared virus and those that did not, but also the “transient” group. This group does mount an early CD4⁺ response against HCV, but it is not sustained. The clinical phenomenon of transient control is common in human infection, with “yo-yo” patterns often described, and will be discussed further below.

All human studies in acute disease have been performed on blood. In these, overall, acute resolving HCV infection has been associated with a sustained response by HCV-specific CD4⁺ T cells. In one representative study patients who failed to clear the virus were divided into two groups^[4]. Group 1 was unable to mount an HCV-specific CD4⁺ T cell response and developed chronic HCV. In Group 2, HCV RNA was cleared initially and was associated with strong HCV-specific CD4⁺ T cell responses. However, these responses diminished just before a rebound of viraemia that resulted in chronic infection. Therefore, a vigorous anti-viral CD4⁺ T cell response (as measured by proliferation assays) in the early and late phase of acute HCV seems necessary to achieve long-term viral control (CD4⁺/Th1 response). However, it is not sufficient (as in the chimpanzees), since those who mount such a response do not necessarily go on to clear virus.

Although what the Gerlach paper described is especially clear cut in relation to the “yo-yo” pattern, other groups

have also reported that the vigour of the T cell response during the early stages of infection may be a critical determinant of disease resolution and control of infection^[5]. That persistent infection can develop despite the presence of acute-phase HCV-specific CD4⁺ T cell responses has been shown in a study of healthcare workers exposed to needle-stick injuries^[6]: although two individuals had strong HCV-specific CD4⁺ T cell proliferative responses in the acute phase with significant decreases in HCV RNA initially, they subsequently became chronically infected. This was an important study since the individuals were available for analysis shortly after infection-before clinical symptoms arose. Since responses are sometimes transient, it may be that in studies where patients are only analysed after they present clinically with acute infection, early T cell responses have been missed.

Such analyses have traditionally relied on proliferation. Using alternative techniques, it has been shown that evolution of the infection to chronicity can be associated with HCV-specific CD4⁺ T cells which survive initially despite failing to proliferate or produce IFN- γ : these diminish eventually as infection persists^[7]. A study of a single patient using a novel approach with Class II MHC peptide complexes (“tetramers”) revealed the loss of functionality of cells before the final loss of detection of such populations^[8]. The failure of such responses in this patient occurred before the re-emergence of virus after transient control. This is important since it suggests that not all the failure of CD4⁺ T cell responses may occur as a consequence of prolonged viremia.

Overall, these studies suggest that a range of HCV-specific CD4⁺ T cell responses in the acute HCV phase can exist in blood and liver. In a subgroup of individuals, it may be that CD4⁺ T cell responses are not mounted initially, or are not detectable at the time of presentation. These individuals are very likely to develop chronic infection^[9]. In another group, strong responses are detected and sustained, which is typically associated with resolution of infection. An important third group (probably the majority of patients) do mount a CD4⁺ T cell response initially, but loss of such responses is associated with progression to chronicity. What the differences are in these initial responses to HCV and why certain key CD4⁺ T cell responses are not sustained remain important questions in understanding HCV persistence. To understand this further it is necessary to consider the exact targets of the CD4⁺ T cell response.

TARGETS OF THE CD4⁺ T CELL RESPONSES IN HCV INFECTION

A more effective immune response against HCV could result from targeting more epitopes, mounting larger responses or targeting a key region. There is some evidence for all three. Overall, permanent resolution of infection has been related to both the breadth (number of CD4 epitopes targeted) and the magnitude of HCV-specific CD4⁺ T cell responses. While no one target has been identified as the key determinant, a number of candidates have been proposed.

Table 1 Displays the most commonly described CD4+ restricted epitopes

Amino acid position	HCV protein	Amino acid sequence	HLA-restriction	Reference
aa 21-40	Core	DVKFPGGGQIVGGVYLLPRR	DRB1*1101,DQB1*0301	Day, 2002
aa 31-45	Core	VGGVYLLPRR GPRLG	DRB1*1101	Godkin, 2001
aa 141-155	Core	GAPLGGAARA LAHGV	DRB1*1101	Godkin, 2001
aa 393-410	E2	GFATQRLTSLFALGPSQK	DRB1*1101	Frasca, 1999
aa 1241-1260	NS3	PAAYAAQGYKVLVLPNSVAA	DRB1*15, DRB1*0301 +	Day, 2002
aa 1248-1261	NS3	GYKVLVLPNSVAAT	DR4, DRB1*1101	Wertheimer, 2003
aa 1248-1267	NS3	GYKVLVLPNSVAATLGFAY	DQB1*0301	Lamonaca, 1999
aa 1251-1259	NS3	VLVLPNSVA	DRB1*1101,DRB1*1201, DRB1*0401, DRB1*1302	Day, 2002
aa 1384-1401	NS3	VIKGGRHILFCHSKKKCD	DRB1*15	Eckels, 1999
aa 1411-1426	NS3	GINAVAYYRGLDVSVI	DRB1*15	Gerlach, 2005
aa 1531-1550	NS3	TPAETTTRLRAYMNTPLPV	DRB1*0701	Day, 2002
aa 1539-1554	NS3	LRAYMNTPLPVCQDH	DRB1*15	Gerlach, 2005
aa 1581-1600	NS3	ENLPYLVAAYQATVCARAQAP	DRB1*1001	Day, 2002
aa 1686-1705	NS4a	VVLSGKPAIIPDREVLREF	DRB1*0301	Harcourt, 2003
aa 1746-1765	NS4b	IAPAVQITNWQKLETFWAKHM	DRB1*16 or DRB3*0202	Harcourt, 2003
aa 1767-1786	NS4b	NFISGIQYLAGLSTLPGNPA	DRB1*1104	Carlos, 2004
aa 1771-1790	NS4b	GIQYLAGLSTLPGNPAIASL	DRB1*0404	Day, 2002
aa 1806-1818	NS4b	TLLFNILGGWVAA	DRB1*0101	Gerlach, 2005
aa 1907-1926	NS4b	GPGEAGAVOWMNRIFAASRG	DRB1*1104,DQB1*0501	Lamonaca, 1999
aa 2268-2282	NS5a	VSVPAEILRK SRRFA	DRB1*1101	Godkin, 2001
aa 2571-2590	NS5b	KGGRKPARLIVFPDLGVRVC	DRB1*0404,DRB1*0407	Day, 2002
aa 2841-2860	NS5b	ARMILMTHFFSVLIARDQLE	DRB1*1101	Day, 2002
aa 2941-2955	NS5b	CGKYLFNWAV RTKLK	DRB1*1101	Godkin, 2001
aa 2941-2960	NS5b	CGKYLFNWAVRTKLKLTPIA	DRB1*1101	Day, 2002

The NS3 protein has been shown to be one dominant target of CD4+ T cell responses in humans clearing HCV infection^[10]. Some epitopes in NS3 have been identified in both humans and chimps with resolved infection (see below)^[11-14]. Numerous studies indicate that CD4+ T cells targeting most HCV proteins, including non-structural (NS) proteins, are the norm in self-limited infections^[3-6,10-11,15-17]. However, some caution is needed in interpreting the exact targeting as the genomic variation between different HCV genotypes is substantial and relatively non-conserved epitopes (as might occur in envelope genes) may not always be picked up in such screens.

The number of CD4+ T cell epitopes recognised during acute HCV infection has been estimated by characterising memory CD4+ T cell populations in blood after permanent resolution of the virus: one study showed at least four, and up to 14 epitopes from the core, NS3, NS4 and NS5 proteins were recognised by CD4+ T cells in patients several months or even years after loss of HCV RNA^[11]. A recent study identified 13 CD4+ T cell epitopes within the NS3-NS4 region that were recognised by $\geq 30\%$ of patients with acute or resolved HCV^[18]. Of these, eight peptides were also recognised recurrently from different donors by specific CD4+ T cell clones in independent cloning procedures. Multispecific CD4+ T cell responses were also detectable in blood of individuals during acute HCV infection acquired by needlestick injury^[6] or IV drug use^[15]. Importantly, in some patients whose infection became chronic after the acute phase infection, responses were similar to those who spontaneously cleared the virus after acute infection^[6]. The only difference (as above) was that these responses in individuals with chronic infection were not sustained.

A recent study identified a single epitope restricted

by HLA DR1 (a common HLA molecule) in NS4, which appears to be very commonly targeted in infected individuals^[18] (Table 1). New analysis using Class II tetramers revealed that in acute infection, all DR1+ donors made a response to this peptide^[19]. This highly reproducible response is somewhat unusual, but may reflect the fact that this peptide is extremely conserved. Although the responses at presentation were indistinguishable between those who went on to resolve infection or not, early loss of tetramer+ cells was seen in those with persistent infection. The loss of such cells was not associated with mutation within the epitope and populations of such cells were not found localised in the liver only. Interestingly, when compared to controls in long term chronic infection, some very low level tetramer+ populations could be identified in the absence of proliferative responses. This suggests that specific responses were not deleted entirely, but persist at very low levels and with only limited function.

Table 1 HCV T helper epitopes mapped within a region of 21 amino acids or less. The protein, the sequence and HLA restriction elements of the T helper epitopes are provided.

ASSOCIATION BETWEEN HLA CLASS II ALLELES AND INFECTION OUTCOME

It is not yet clear whether responses to specific epitopes are clearly related to outcome. However, the T cell response to peptides derived from the virus is directed by the MHC genotype, and this could have a major influence on the quality of T cell responses. Importantly, some MHC Class II alleles in humans have been associated with persistence or resolution of HCV infection. For example, HLA-DRB1*0701 has been shown to be associated with

persistence in patients who were homogeneous in terms of gender, source of infection (genotype 1b) and ethnicity^[20]. In contrast, a number of other studies of more mixed populations have shown a strong association between other HLA Class II alleles and viral control. These alleles are HLA-DRB1*0101, HLA-DRB1*1101, and HLA-DQB1*0301^[21-23].

Although specific HLA alleles have been defined as protective, the link between the possession of a specific allele and the presentation of a specific peptide or set of peptides has not yet been made. HLA-DRB1*1101 and HLA-DQB1*0301 (these genes are in close linkage disequilibrium) have been associated with a sustained CD4⁺ T cell response in resolved HCV infection; these responses were stronger than in non-DQB1*0301+ controls^[24]. However, our understanding of this association still remains incomplete. Some of these HLA Class II restricted peptides have been identified through epitope prediction programs^[25], and the full repertoire of naturally presented peptides is not completely defined. A recent study showed that viral variation might play a role in determining the dominance of epitopes seen within a population. Here, an HLA DR11-restricted epitope (NS3 aa1248-1261) that is highly conserved within viral genotypes was found not to be the immunodominant response, despite being the most commonly recognised epitope for this HLA allele^[26]. Epitopes in viral regions that can tolerate amino acid substitutions may thus appear to be less dominant, even if they are important. This is because their capacity for increased variability means that they may not occur in the prototype viral peptide sequences often used for immunological study, or might only be represented in a fraction of the patients studied.

CD4⁺ T CELL QUALITY AND ASSOCIATION WITH ACUTE OUTCOME

It has been suggested in studies of acute disease that viral clearance is more likely to occur when HCV specific responses of patients display a Th1 profile (IFN- γ and IL-2). Those with a more typical Th2 profile (IL-4 and IL-10) were more likely to become chronic^[27] suggesting that the Th1 phenotype generates more protective immune responses in HCV. In that study, CD4⁺ T-cell proliferation and cytokine secretion in response to a panel of recombinant HCV antigens were assayed in 17 patients with acute HCV. All six patients with self-limited disease had a significant CD4⁺ T-cell proliferation to C22, E1, C100, C200, and NS5, running parallel with the antigen-stimulated secretion of IL-2 and IFN- γ , but not with IL-4 and IL-10, indicating predominant Th1 responses. Among the remaining 11 patients who developed chronicity, several cases showed specific CD4⁺ T cell responses, but their antigen-stimulated IL-2 and IFN- γ production were significantly lower than those of cases with recovery. Importantly, IL-4 and IL-10 (Th2 responses) were detectable in the group who developed chronicity. The data suggested that activation of Th2 responses in acute hepatitis C patients might play a role in the development

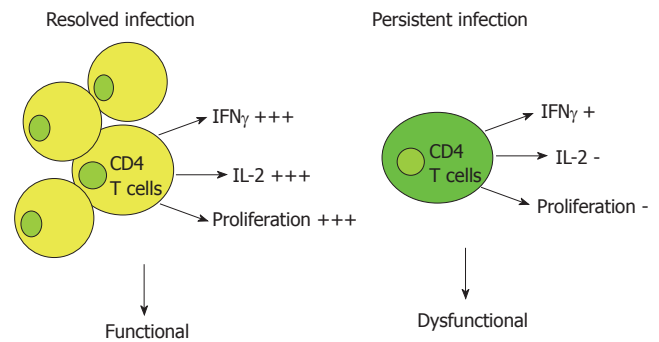


Figure 1 Cytokine secretion patterns in persistent versus resolved HCV infection.

of chronicity, but much more work needs to be done in this area, and why such responses might differ at the start of the disease needs some explanation.

HCV SPECIFIC RESPONSES IN CHRONIC INFECTION

As discussed above, once chronic HCV infection is established, cellular immune responses are rarely or barely detectable using current technology. Several groups have aimed to analyse these HCV-specific CD4⁺ T cell responses during chronic HCV infection using proliferation assays, cytokine assays and, more recently Class II tetramers.

Analysis of HCV-specific CD4⁺ T cell responses in chronic HCV infection using ELISpot or ICS showed responses at low frequency in blood and only targeted a limited number of epitopes^[4,7,11,15-17,28]. In an expanded analysis of responses to recombinant HCV proteins in persons with resolved infection, an average of 10 epitopes was targeted, whereas in persons with chronic viraemia never more than one epitope was targeted^[11].

The question of whether HCV-specific CD4⁺ T cell responses persist in chronic infection but lack function in terms of proliferation and cytokine production (IFN- γ) has been addressed by staining for expression of the IL-2 receptor α -chain (CD25), which is an early marker of activation in a study that showed that a small proportion of CD4⁺ T cells from the blood of chronically infected individuals did upregulate CD25 after stimulation with recombinant HCV proteins, but lacked the capability for proliferation and IFN- γ production^[7]. However, the use of the marker CD25, which is present normally on a fraction of CD4⁺ T cells including Tregs, limits the usefulness of this assay.

It has generally been assumed that loss of proliferation equates to loss of the specific T cell populations. Low levels of IL-2 secretion have been shown in independent studies in humans and mice to be accompanied by a loss of proliferative capacity both *in vitro* and *in vivo*^[29-32]. In HCV infection, recent data suggest that, in the presence of viraemia, HCV-specific CD4⁺ T cell populations do exist but lack proliferative capacity^[33]. This status is associated with the production of IFN- γ upon antigen stimulation, but little or no IL-2 is expressed^[34] (Figure 1).

The best approach for detecting functionally impaired

CD4⁺ T cells in chronic HCV infection is the use of MHC Class II tetramers. Using this technique, a correlation has been shown between the clinical outcome and the presence of circulating CD4⁺ T cells directed against the virus^[35]. Here, with the use of 3 HCV HLA Class II tetramers, HCV-specific CD4⁺ T cells could be detected in subjects who spontaneously resolved HCV viraemia, but not in those with chronic HCV infection, suggesting that HCV-specific CD4⁺ T cell frequencies are very low in PBMC. A further application of this technology for the analysis of intrahepatic CD4⁺ T cells could shed important light on their differentiation state and functionality.

The expansion of HCV-specific CD4⁺ T cell lines by repeated stimulation with recombinant antigens indicated that antigen-specific populations do persist^[36-38]. A note of caution should be injected here in the analysis of sustained CD4⁺ T cell responses in chronically infected patients. In many cases, these may represent a historical response to a previous viral genotype which is no longer circulating in the patient. In one case described, the response was directed against genotype 1 even though the patient carried genotype 3, with a historical genotype 1 infection^[39]. Since the peptide in the genotype 3 sequence was substantially different and not recognised by host CD4⁺ T cells, this indicates that the detected response is effectively a memory response after removal of the original virus. Since superinfection is relatively common, this issue may be a substantial one for both CD4⁺ and CD8⁺ T cells^[40].

The role of IL-10 as Th2 anti-inflammatory cytokine has been demonstrated *in vivo* in humans chronically infected with HCV^[41]. Here, individuals with advanced fibrosis were treated three times a week with IL-10 for 12 mo. Administration of IL-10 resulted in a decreased number of IFN- γ -secreting HCV-specific CD4⁺ and CD8⁺ T cells. At the same time ALT levels as a marker of inflammation were reduced, indicating the role of IL-10 as anti-inflammatory cytokine. However, with the loss of specific CD4⁺ and CD8⁺ T cells, HCV RNA levels were increased, suggesting that these same cells are responsible for viral control^[41].

One hypothesis why in chronic HCV the Th2 type may occur is that dendritic cells from patients with chronic HCV infection have defective function, possibly due to inhibition of IL-12^[42,43]. The latter cytokine is required for the induction of Th1 type cells. This dendritic cell dysfunction might result in biased T cell polarisation which could favor, for example, a Th2-type response. However, to what extent this is a major factor in pathogenesis is still controversial and the findings are not uniformly reproducible.

Overall, failure of CD4⁺ T cells is a key factor in HCV persistence and clearly in chronic disease there are relatively few functional CD4⁺ T cells to find, by whatever method. To some extent this appears to be due to loss/deletion of antigen-specific cells. On the other hand there is some evidence that a change in function also occurs in persistent infection, although whether this is cause or effect requires a great deal more study. It should be noted that although CD4⁺ T cell responses are regarded as weak in chronic HCV mono-infection, they are even weaker in HCV/HIV co-infection^[44,45]. Since co-infection is associated with an

increase of HCV load of about 0.5-1 log, this data suggests that in chronic mono-infection the remaining CD4⁺ T cell response is still playing a significant role.

MODELS FOR FAILURE OF CD4⁺ T CELL RESPONSES

If failure of the CD4⁺ T cell response against HCV is associated with virus persistence, what mechanisms could account for this? Here we outline three major contenders, escape, exhaustion and regulation.

Escape through mutation

Numerous studies in both animal and human models have documented immune escape from virus-specific CTL responses by viral mutations in CTL epitopes that lead to loss of immune control and viral persistence^[46-52]. Less information is currently available about the potential for immune escape from viral CD4⁺ T cell epitopes, although limited studies in chronic HIV and HCV infection have identified multiple autologous virus variants for specific CD4⁺ T cell epitopes^[53-56]. Peptides corresponding to viral variants were synthesised and tested in *in vitro* assays, and the majority of variants failed to stimulate proliferation or cytokine production by CD4⁺ T cells^[54-56]. That viral variants may play a role in HCV persistence has also been shown previously in a study with four HLA-DRB1*15 patients chronically infected with HCV^[57]. Here, naturally occurring single amino acid substitutions in the DRB1*15-restricted Th1 epitope (aa 358-375) in the NS3 protein failed to stimulate proliferation. This was also accompanied by a shift in cytokine secretion patterns from one characteristic of a Th1 anti-viral response to a Th2 form. These data suggest that viral immune escape from specific CD4⁺ T cell responses is possible, but clear data showing the evolution of CD4⁺ T cell escape mutants in response to T cell selection pressure are still needed.

A recent study analyzed the effects of an induced T-cell response in three immunized chimpanzees, targeting nonstructural proteins in the absence of neutralizing antibodies^[58]. The immunized animals were challenged with clonal HCV, which had the same sequence as the antigens used for immunization. Persistent control of the virus was observed in two animals, whereas in the third animal viral control was transient, followed by a resurgence concomitant with the emergence of new dominant viral populations bearing single amino acid changes in the NS3 and NS5A regions. These mutations resulted in a loss of CD4 T-cell recognition and subsequent to viral resurgence and immune escape a large fraction of NS3-specific T cells became impaired in their ability to secrete IFN- γ and proliferate.

Exhaustion

In addition to escape from virus-specific T cell responses, escape from neutralising antibody (nAb) responses is thought to be one potential mechanism leading to the persistence of some viruses with knock on effects on CD4⁺ T cells^[53,59,60]. Recent data generated in an LCMV model have provided additional insight into the relationship between

CD4⁺ T cells and immune escape from nAb responses. CD8^{-/-} mice were infected with the WE strain of LCMV to establish a long-term infection with high levels of virus production that is transiently controlled by nAbs^[60]. However, the lack of CD8⁺ CTL responses and consequently high viraemia in this model leads to escape from polyclonal nAb responses. Associated with this is the rapid induction of CD4⁺ T cell unresponsiveness^[61]. Although the molecular mechanism of CD4⁺ T cell unresponsiveness is not clear from this study, it has been postulated that the high antigenic load in this model system may have resulted in activation of all virus-specific CD4⁺ T cells, leading to exhaustion and activation-induced cell death as has been described for CTLs^[62,63]. Importantly, in the absence of LCMV-specific CD4⁺ T cells, these mice failed to generate new effective humoral responses against emerging neutralisation-escape mutants and the viral infection persisted^[61]. These data provide further evidence for the importance of interactions between the cellular and humoral immune responses for efficient control of viral infections. A similar phenomenon of exhaustion of CD4⁺ T cells could easily arise through any mechanism, which leads to long-term viremia, including escape from interferon or mutation in epitopes recognised by CD8⁺ T cells.

Regulation

Recent years have seen a revival of interest in the role of regulatory T cells—notably the CD4⁺ CD25⁺ FoxP3⁺ subset^[64]. It is plausible that in HCV infection excessive regulation is involved in the suppression of HCV specific T-cell responses. Recently, CD4⁺ CD25⁺ regulatory T-cell activity has been shown to be present in patients with chronic HCV infection, which may contribute to weak HCV-specific T-cell responses and viral persistence^[65-69]. An important question that derives from these studies is to what extent the Treg activity seen in persistent infection relates to the activity of antigen-specific cells. Treg cells may arise in the thymus (natural Tregs) but additionally virus-specific cells, which are repetitively stimulated with antigen over time, may develop regulatory characteristics. This activity may be promoted by the action of dendritic cell subsets modulated by persistent viremia. Such Treg cells might serve to downregulate both CD4⁺ and CD8⁺ T cell responses in persistent infection, particularly within the inflamed liver. A recent study revealed that approximately one in two to one in three CD4⁺ T cells in the liver of chronically infected patients are FoxP3⁺, a remarkably dominant potential Treg population^[70]. This could have a major effect on the maintenance and growth of antigen-specific CD4⁺ and CD8⁺ T cells.

CONCLUSION

HCV specific CD4⁺ T cells hold a pivotal role in disease pathogenesis. There is a consensus that there are real differences between the responses seen in resolved infection vs persistent infection, although to what extent these are cause or consequence is not clear yet. The differences include not only number, but also function, including cytokine secretion, such as the key mediator IL-2. A number of pieces of evidence point to the fact that a robust CD4⁺

T cell response is associated with a good outcome from acute infection and there is no doubt that such responses should be elicited in a vaccine. To what extent it matters which epitopes are targeted or not is not yet clear, but most observers argue that breadth is important, especially given the huge genomic variation in HCV, and that numbers are important. For those lucky enough to inherit protective HLA types, a vaccine may simply augment an already favourable response and sustain adaptive responses from CD8⁺ and B cells. For the rest, perhaps a pool of CD4⁺ T cell responses which are primed in a normal uninfected individual, and which can expand rapidly during acute infection may be sufficient to tip the balance in favour of host clearance.

REFERENCES

- 1 Gremion C, Cerny A. Hepatitis C virus and the immune system: a concise review. *Rev Med Virol* 2005; **15**: 235-268
- 2 Grakoui A, Shoukry NH, Woollard DJ, Han JH, Hanson HL, Ghayeb J, Murthy KK, Rice CM, Walker CM. HCV persistence and immune evasion in the absence of memory T cell help. *Science* 2003; **302**: 659-662
- 3 Thimme R, Bukh J, Spangenberg HC, Wieland S, Pemberton J, Steiger C, Govindarajan S, Purcell RH, Chisari FV. Viral and immunological determinants of hepatitis C virus clearance, persistence, and disease. *Proc Natl Acad Sci USA* 2002; **99**: 15661-15668
- 4 Gerlach JT, Diepolder HM, Jung MC, Gruener NH, Schraut WW, Zachoval R, Hoffmann R, Schirren CA, Santantonio T, Pape GR. Recurrence of hepatitis C virus after loss of virus-specific CD4(+) T-cell response in acute hepatitis C. *Gastroenterology* 1999; **117**: 933-941
- 5 Missale G, Bertoni R, Lamonaca V, Valli A, Massari M, Mori C, Rumi MG, Houghton M, Fiaccadori F, Ferrari C. Different clinical behaviors of acute hepatitis C virus infection are associated with different vigor of the anti-viral cell-mediated immune response. *J Clin Invest* 1996; **98**: 706-714
- 6 Thimme R, Oldach D, Chang KM, Steiger C, Ray SC, Chisari FV. Determinants of viral clearance and persistence during acute hepatitis C virus infection. *J Exp Med* 2001; **194**: 1395-1406
- 7 Ulsenheimer A, Gerlach JT, Gruener NH, Jung MC, Schirren CA, Schraut W, Zachoval R, Pape GR, Diepolder HM. Detection of functionally altered hepatitis C virus-specific CD4 T cells in acute and chronic hepatitis C. *Hepatology* 2003; **37**: 1189-1198
- 8 Ulsenheimer A, Lucas M, Seth NP, Tilman Gerlach J, Gruener NH, Loughry A, Pape GR, Wucherpfennig KW, Diepolder HM, Klennerman P. Transient immunological control during acute hepatitis C virus infection: ex vivo analysis of helper T-cell responses. *J Viral Hepat* 2006; **13**: 708-714
- 9 Aberle JH, Formann E, Steindl-Munda P, Weseslindtner L, Gurguta C, Perstinger G, Grilnberger E, Laferl H, Dienes HP, Popow-Kraupp T, Ferenci P, Holzmann H. Prospective study of viral clearance and CD4(+) T-cell response in acute hepatitis C primary infection and reinfection. *J Clin Virol* 2006; **36**: 24-31
- 10 Diepolder HM, Zachoval R, Hoffmann RM, Wierenga EA, Santantonio T, Jung MC, Eichenlaub D, Pape GR. Possible mechanism involving T-lymphocyte response to non-structural protein 3 in viral clearance in acute hepatitis C virus infection. *Lancet* 1995; **346**: 1006-1007
- 11 Day CL, Lauer GM, Robbins GK, McGovern B, Wurcel AG, Gandhi RT, Chung RT, Walker BD. Broad specificity of virus-specific CD4⁺ T-helper-cell responses in resolved hepatitis C virus infection. *J Virol* 2002; **76**: 12584-12595
- 12 Lamonaca V, Missale G, Urbani S, Pilli M, Boni C, Mori C, Sette A, Massari M, Southwood S, Bertoni R, Valli A, Fiaccadori F, Ferrari C. Conserved hepatitis C virus sequences are highly immunogenic for CD4(+) T cells: implications for vac-

- cine development. *Hepatology* 1999; **30**: 1088-1098
- 13 **Shoukry NH**, Sidney J, Sette A, Walker CM. Conserved hierarchy of helper T cell responses in a chimpanzee during primary and secondary hepatitis C virus infections. *J Immunol* 2004; **172**: 483-492
 - 14 **Diepolder HM**, Gerlach JT, Zachoval R, Hoffmann RM, Jung MC, Wierenga EA, Scholz S, Santantonio T, Houghton M, Southwood S, Sette A, Pape GR. Immunodominant CD4⁺ T-cell epitope within nonstructural protein 3 in acute hepatitis C virus infection. *J Virol* 1997; **71**: 6011-6019
 - 15 **Lechner F**, Wong DK, Dunbar PR, Chapman R, Chung RT, Dohrenwend P, Robbins G, Phillips R, Klenerman P, Walker BD. Analysis of successful immune responses in persons infected with hepatitis C virus. *J Exp Med* 2000; **191**: 1499-1512
 - 16 **Rosen HR**, Miner C, Sasaki AW, Lewinsohn DM, Conrad AJ, Bakke A, Bouwer HG, Hinrichs DJ. Frequencies of HCV-specific effector CD4⁺ T cells by flow cytometry: correlation with clinical disease stages. *Hepatology* 2002; **35**: 190-198
 - 17 **Takaki A**, Wiese M, Maertens G, Depla E, Seifert U, Liebetrau A, Miller JL, Manns MP, Reherrmann B. Cellular immune responses persist and humoral responses decrease two decades after recovery from a single-source outbreak of hepatitis C. *Nat Med* 2000; **6**: 578-582
 - 18 **Gerlach JT**, Ulsenheimer A, Grüner NH, Jung MC, Schraut W, Schirren CA, Heeg M, Scholz S, Witter K, Zahn R, Vogler A, Zachoval R, Pape GR, Diepolder HM. Minimal T-cell-stimulatory sequences and spectrum of HLA restriction of immunodominant CD4⁺ T-cell epitopes within hepatitis C virus NS3 and NS4 proteins. *J Virol* 2005; **79**: 12425-12433
 - 19 **Lucas M**, Ulsenheimer A, Pfaffert K, Heeg MH, Gaudieri S, Grüner N, Rauch A, Gerlach JT, Jung MC, Zachoval R, Pape GR, Schraut W, Santantonio T, Nitschko H, Obermeier M, Phillips R, Scriba TJ, Semmo N, Day C, Weber JN, Fidler S, Thimme R, Haberstroh A, Baumert TF, Klenerman P, Diepolder HM. Tracking virus-specific CD4⁺ T cells during and after acute hepatitis C virus infection. *PLoS ONE* 2007; **2**: e649
 - 20 **Fanning LJ**, Levis J, Kenny-Walsh E, Whelton M, O'Sullivan K, Shanahan F. HLA class II genes determine the natural variance of hepatitis C viral load. *Hepatology* 2001; **33**: 224-230
 - 21 **Alric L**, Fort M, Izopet J, Vinel JP, Charlet JP, Selves J, Puel J, Pascal JP, Duffaut M, Abbal M. Genes of the major histocompatibility complex class II influence the outcome of hepatitis C virus infection. *Gastroenterology* 1997; **113**: 1675-1681
 - 22 **Minton EJ**, Smillie D, Neal KR, Irving WL, Underwood JC, James V. Association between MHC class II alleles and clearance of circulating hepatitis C virus. Members of the Trent Hepatitis C Virus Study Group. *J Infect Dis* 1998; **178**: 39-44
 - 23 **Thursz M**, Yallop R, Goldin R, Trepo C, Thomas HC. Influence of MHC class II genotype on outcome of infection with hepatitis C virus. The HENCORE group. Hepatitis C European Network for Cooperative Research. *Lancet* 1999; **354**: 2119-2124
 - 24 **Harcourt G**, Hellier S, Bunce M, Satsangi J, Collier J, Chapman R, Phillips R, Klenerman P. Effect of HLA class II genotype on T helper lymphocyte responses and viral control in hepatitis C virus infection. *J Viral Hepat* 2001; **8**: 174-179
 - 25 **Godkin A**, Jeanguet N, Thursz M, Openshaw P, Thomas H. Characterization of novel HLA-DR11-restricted HCV epitopes reveals both qualitative and quantitative differences in HCV-specific CD4⁺ T cell responses in chronically infected and non-viremic patients. *Eur J Immunol* 2001; **31**: 1438-1446
 - 26 **Harcourt GC**, Lucas M, Sheridan I, Barnes E, Phillips R, Klenerman P. Longitudinal mapping of protective CD4⁺ T cell responses against HCV: analysis of fluctuating dominant and subdominant HLA-DR11 restricted epitopes. *J Viral Hepat* 2004; **11**: 324-331
 - 27 **Tsai SL**, Liaw YF, Chen MH, Huang CY, Kuo GC. Detection of type 2-like T-helper cells in hepatitis C virus infection: implications for hepatitis C virus chronicity. *Hepatology* 1997; **25**: 449-458
 - 28 **Wertheimer AM**, Miner C, Lewinsohn DM, Sasaki AW, Kaufman E, Rosen HR. Novel CD4⁺ and CD8⁺ T-cell determinants within the NS3 protein in subjects with spontaneously resolved HCV infection. *Hepatology* 2003; **37**: 577-589
 - 29 **Younes SA**, Yassine-Diab B, Dumont AR, Boulassel MR, Grossman Z, Routy JP, Sekaly RP. HIV-1 viremia prevents the establishment of interleukin 2-producing HIV-specific memory CD4⁺ T cells endowed with proliferative capacity. *J Exp Med* 2003; **198**: 1909-1922
 - 30 **Iyassere C**, Tilton JC, Johnson AJ, Younes S, Yassine-Diab B, Sekaly RP, Kwok WW, Migueles SA, Laborico AC, Shupert WL, Hallahan CW, Davey RT Jr, Dybul M, Vogel S, Metcalf J, Connors M. Diminished proliferation of human immunodeficiency virus-specific CD4⁺ T cells is associated with diminished interleukin-2 (IL-2) production and is recovered by exogenous IL-2. *J Virol* 2003; **77**: 10900-10909
 - 31 **Wherry EJ**, Teichgräber V, Becker TC, Masopust D, Kaech SM, Antia R, von Andrian UH, Ahmed R. Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat Immunol* 2003; **4**: 225-234
 - 32 **Fuller MJ**, Zajac AJ. Ablation of CD8 and CD4 T cell responses by high viral loads. *J Immunol* 2003; **170**: 477-486
 - 33 **Semmo N**, Krashias G, Willberg C, Klenerman P. Analysis of the relationship between cytokine secretion and proliferative capacity in hepatitis C virus infection. *J Viral Hepat* 2007; **14**: 492-502
 - 34 **Semmo N**, Day CL, Ward SM, Lucas M, Harcourt G, Loughry A, Klenerman P. Preferential loss of IL-2-secreting CD4⁺ T helper cells in chronic HCV infection. *Hepatology* 2005; **41**: 1019-1028
 - 35 **Day CL**, Seth NP, Lucas M, Appel H, Gauthier L, Lauer GM, Robbins GK, Szczepiorkowski ZM, Casson DR, Chung RT, Bell S, Harcourt G, Walker BD, Klenerman P, Wucherpfennig KW. Ex vivo analysis of human memory CD4 T cells specific for hepatitis C virus using MHC class II tetramers. *J Clin Invest* 2003; **112**: 831-842
 - 36 **Minutello MA**, Pileri P, Unutmaz D, Censini S, Kuo G, Houghton M, Brunetto MR, Bonino F, Abrignani S. Compartmentalization of T lymphocytes to the site of disease: intrahepatic CD4⁺ T cells specific for the protein NS4 of hepatitis C virus in patients with chronic hepatitis C. *J Exp Med* 1993; **178**: 17-25
 - 37 **Penna A**, Missale G, Lamonaca V, Pilli M, Mori C, Zanelli P, Cavalli A, Elia G, Ferrari C. Intrahepatic and circulating HLA class II-restricted, hepatitis C virus-specific T cells: functional characterization in patients with chronic hepatitis C. *Hepatology* 2002; **35**: 1225-1236
 - 38 **Schirren CA**, Jung MC, Gerlach JT, Worzfeld T, Baretton G, Mamin M, Hubert Gruener N, Houghton M, Pape GR. Liver-derived hepatitis C virus (HCV)-specific CD4(+) T cells recognize multiple HCV epitopes and produce interferon gamma. *Hepatology* 2000; **32**: 597-603
 - 39 **Harcourt GC**, Lucas M, Godkin AJ, Kantzanou M, Phillips RE, Klenerman P. Evidence for lack of cross-genotype protection of CD4⁺ T cell responses during chronic hepatitis C virus infection. *Clin Exp Immunol* 2003; **131**: 122-129
 - 40 **Lauer GM**, Barnes E, Lucas M, Timm J, Ouchi K, Kim AY, Day CL, Robbins GK, Casson DR, Reiser M, Dusheiko G, Allen TM, Chung RT, Walker BD, Klenerman P. High resolution analysis of cellular immune responses in resolved and persistent hepatitis C virus infection. *Gastroenterology* 2004; **127**: 924-936
 - 41 **Nelson DR**, Tu Z, Soldevila-Pico C, Abdelmalek M, Zhu H, Xu YL, Cabrera R, Liu C, Davis GL. Long-term interleukin 10 therapy in chronic hepatitis C patients has a proviral and anti-inflammatory effect. *Hepatology* 2003; **38**: 859-868
 - 42 **Bain C**, Fatmi A, Zoulim F, Zarski JP, Trépo C, Inchauspé G. Impaired allostimulatory function of dendritic cells in chronic hepatitis C infection. *Gastroenterology* 2001; **120**: 512-524
 - 43 **Fowler NL**, Torresi J, Jackson DC, Brown LE, Gowans EJ. Immune responses in hepatitis C virus infection: the role of dendritic cells. *Immunol Cell Biol* 2003; **81**: 63-66
 - 44 **Lauer GM**, Nguyen TN, Day CL, Robbins GK, Flynn T, McGowan K, Rosenberg ES, Lucas M, Klenerman P, Chung RT, Walker BD. Human immunodeficiency virus type 1-hepatitis C virus coinfection: intraindividual comparison of cellular immune responses against two persistent viruses. *J Virol* 2002; **76**: 2817-2826

- 45 **Harcourt G**, Gomperts E, Donfield S, Klenerman P. Diminished frequency of hepatitis C virus specific interferon {gamma} secreting CD4+ T cells in human immunodeficiency virus/hepatitis C virus coinfecting patients. *Gut* 2006; **55**: 1484-1487
- 46 **Erickson AL**, Kimura Y, Igarashi S, Eichelberger J, Houghton M, Sidney J, McKinney D, Sette A, Hughes AL, Walker CM. The outcome of hepatitis C virus infection is predicted by escape mutations in epitopes targeted by cytotoxic T lymphocytes. *Immunity* 2001; **15**: 883-895
- 47 **Vogel TU**, Friedrich TC, O'Connor DH, Rehrauer W, Dodds EJ, Hickman H, Hildebrand W, Sidney J, Sette A, Hughes A, Horton H, Vielhuber K, Rudersdorf R, De Souza IP, Reynolds MR, Allen TM, Wilson N, Watkins DI. Escape in one of two cytotoxic T-lymphocyte epitopes bound by a high-frequency major histocompatibility complex class I molecule, Mamu-A*02: a paradigm for virus evolution and persistence? *J Virol* 2002; **76**: 11623-11636
- 48 **O'Connor DH**, Allen TM, Vogel TU, Jing P, DeSouza IP, Dodds E, Dunphy EJ, Melsaether C, Mothé B, Yamamoto H, Horton H, Wilson N, Hughes AL, Watkins DI. Acute phase cytotoxic T lymphocyte escape is a hallmark of simian immunodeficiency virus infection. *Nat Med* 2002; **8**: 493-499
- 49 **Allen TM**, O'Connor DH, Jing P, Dzuris JL, Mothé BR, Vogel TU, Dunphy E, Liebl ME, Emerson C, Wilson N, Kunstman KJ, Wang X, Allison DB, Hughes AL, Desrosiers RC, Altman JD, Wolinsky SM, Sette A, Watkins DI. Tat-specific cytotoxic T lymphocytes select for SIV escape variants during resolution of primary viraemia. *Nature* 2000; **407**: 386-390
- 50 **Altfeld M**, Allen TM, Yu XG, Johnston MN, Agrawal D, Korber BT, Montefiori DC, O'Connor DH, Davis BT, Lee PK, Maier EL, Harlow J, Goulder PJ, Brander C, Rosenberg ES, Walker BD. HIV-1 superinfection despite broad CD8+ T-cell responses containing replication of the primary virus. *Nature* 2002; **420**: 434-439
- 51 **Goulder PJ**, Phillips RE, Colbert RA, McAdam S, Ogg G, Nowak MA, Giangrande P, Luzzi G, Morgan B, Edwards A, McMichael AJ, Rowland-Jones S. Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. *Nat Med* 1997; **3**: 212-217
- 52 **Borrow P**, Lewicki H, Wei X, Horwitz MS, Pfeffer N, Meyers H, Nelson JA, Gairin JE, Hahn BH, Oldstone MB, Shaw GM. Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. *Nat Med* 1997; **3**: 205-211
- 53 **von Hahn T**, Yoon JC, Alter H, Rice CM, Rehmann B, Balfe P, McKeating JA. Hepatitis C virus continuously escapes from neutralizing antibody and T-cell responses during chronic infection in vivo. *Gastroenterology* 2007; **132**: 667-678
- 54 **Wang H**, Eckels DD. Mutations in immunodominant T cell epitopes derived from the nonstructural 3 protein of hepatitis C virus have the potential for generating escape variants that may have important consequences for T cell recognition. *J Immunol* 1999; **162**: 4177-4183
- 55 **Harcourt GC**, Garrard S, Davenport MP, Edwards A, Phillips RE. HIV-1 variation diminishes CD4 T lymphocyte recognition. *J Exp Med* 1998; **188**: 1785-1793
- 56 **Eckels DD**, Zhou H, Bian TH, Wang H. Identification of antigenic escape variants in an immunodominant epitope of hepatitis C virus. *Int Immunol* 1999; **11**: 577-583
- 57 **Wang JH**, Layden TJ, Eckels DD. Modulation of the peripheral T-Cell response by CD4 mutants of hepatitis C virus: transition from a Th1 to a Th2 response. *Hum Immunol* 2003; **64**: 662-673
- 58 **Puig M**, Mihalik K, Tilton JC, Williams O, Merchinsky M, Connors M, Feinstone SM, Major ME. CD4+ immune escape and subsequent T-cell failure following chimpanzee immunization against hepatitis C virus. *Hepatology* 2006; **44**: 736-745
- 59 **Parren PW**, Moore JP, Burton DR, Sattentau QJ. The neutralizing antibody response to HIV-1: viral evasion and escape from humoral immunity. *AIDS* 1999; **13** Suppl A: S137-S162
- 60 **Ciurea A**, Klenerman P, Hunziker L, Horvath E, Senn BM, Ochsenbein AF, Hengartner H, Zinkernagel RM. Viral persistence in vivo through selection of neutralizing antibody-escape variants. *Proc Natl Acad Sci USA* 2000; **97**: 2749-2754
- 61 **Ciurea A**, Hunziker L, Klenerman P, Hengartner H, Zinkernagel RM. Impairment of CD4(+) T cell responses during chronic virus infection prevents neutralizing antibody responses against virus escape mutants. *J Exp Med* 2001; **193**: 297-305
- 62 **Gallimore A**, Glithero A, Godkin A, Tissot AC, Plückthun A, Elliott T, Hengartner H, Zinkernagel R. Induction and exhaustion of lymphocytic choriomeningitis virus-specific cytotoxic T lymphocytes visualized using soluble tetrameric major histocompatibility complex class I-peptide complexes. *J Exp Med* 1998; **187**: 1383-1393
- 63 **Moskophidis D**, Lechner F, Pircher H, Zinkernagel RM. Virus persistence in acutely infected immunocompetent mice by exhaustion of antiviral cytotoxic effector T cells. *Nature* 1993; **362**: 758-761
- 64 **Rouse BT**, Sarangi PP, Suvas S. Regulatory T cells in virus infections. *Immunol Rev* 2006; **212**: 272-286
- 65 **Rushbrook SM**, Ward SM, Unitt E, Vowler SL, Lucas M, Klenerman P, Alexander GJ. Regulatory T cells suppress in vitro proliferation of virus-specific CD8+ T cells during persistent hepatitis C virus infection. *J Virol* 2005; **79**: 7852-7859
- 66 **Boettler T**, Spangenberg HC, Neumann-Haefelin C, Panther E, Urbani S, Ferrari C, Blum HE, von Weizsäcker F, Thimme R. T cells with a CD4+CD25+ regulatory phenotype suppress in vitro proliferation of virus-specific CD8+ T cells during chronic hepatitis C virus infection. *J Virol* 2005; **79**: 7860-7867
- 67 **Cabrera R**, Tu Z, Xu Y, Firpi RJ, Rosen HR, Liu C, Nelson DR. An immunomodulatory role for CD4(+)CD25(+) regulatory T lymphocytes in hepatitis C virus infection. *Hepatology* 2004; **40**: 1062-1071
- 68 **Sugimoto K**, Ikeda F, Stadanlick J, Nunes FA, Alter HJ, Chang KM. Suppression of HCV-specific T cells without differential hierarchy demonstrated ex vivo in persistent HCV infection. *Hepatology* 2003; **38**: 1437-1448
- 69 **Bolacchi F**, Sinistro A, Ciaprin C, Demin F, Capozzi M, Carducci FC, Drapeau CM, Rocchi G, Bergamini A. Increased hepatitis C virus (HCV)-specific CD4+CD25+ regulatory T lymphocytes and reduced HCV-specific CD4+ T cell response in HCV-infected patients with normal versus abnormal alanine aminotransferase levels. *Clin Exp Immunol* 2006; **144**: 188-196
- 70 **Ward SM**, Fox BC, Brown PJ, Worthington J, Fox SB, Chapman RW, Fleming KA, Banham AH, Klenerman P. Quantification and localisation of FOXP3+ T lymphocytes and relation to hepatic inflammation during chronic HCV infection. *J Hepatol* 2007; **47**: 316-324

S- Editor Ma N L- Editor Roberts SE E- Editor Ma WH

Robert Thimme, MD, Professor, Series Editor

Host and viral factors contributing to CD8+ T cell failure in hepatitis C virus infection

Christoph Neumann-Haefelin, Hans Christian Spangenberg, Hubert E Blum, Robert Thimme

Christoph Neumann-Haefelin, Hans Christian Spangenberg, Hubert E Blum, Robert Thimme, Department of Medicine II, University Hospital Freiburg, Germany

Supported by the Deutsche Forschungsgemeinschaft (Emmy Noether Programm, SFB 610), the Wilhelm Sander Stiftung, and the Bundesministerium fuer Wissenschaft und Forschung (Start-up fonds Kompetenznetz Hepatitis)

Correspondence to: Christoph Neumann-Haefelin, Department of Medicine II, University Hospital Freiburg, Hugstetter Strasse 55, Freiburg D-79106,

Germany. christoph.neumann-haefelin@uniklinik-freiburg.de

Telephone: +49-761-2703401 Fax: +49-761-2703725

Received: June 26, 2007 Revised: July 9, 2007

<http://www.wjgnet.com/1007-9327/13/4839.asp>

Abstract

Virus-specific CD8+ T cells are thought to be the major anti-viral effector cells in hepatitis C virus (HCV) infection. Indeed, viral clearance is associated with vigorous CD8+ T cell responses targeting multiple epitopes. In the chronic phase of infection, HCV-specific CD8+ T cell responses are usually weak, narrowly focused and display often functional defects regarding cytotoxicity, cytokine production, and proliferative capacity. In the last few years, different mechanisms which might contribute to the failure of HCV-specific CD8+ T cells in chronic infection have been identified, including insufficient CD4+ help, deficient CD8+ T cell differentiation, viral escape mutations, suppression by viral factors, inhibitory cytokines, inhibitory ligands, and regulatory T cells. In addition, host genetic factors such as the host's human leukocyte antigen (HLA) background may play an important role in the efficiency of the HCV-specific CD8+ T cell response and thus outcome of infection. The growing understanding of the mechanisms contributing to T cell failure and persistence of HCV infection will contribute to the development of successful immunotherapeutical and -prophylactical strategies.

© 2007 WJG. All rights reserved.

Key words: Hepatitis C virus; CD8+ T cells; T cell failure; Viral escape; Programmed death 1; Regulatory T cells; T cell maturation; Human leukocyte antigen

Neumann-Haefelin C, Spangenberg HC, Blum HE, Thimme R. Host and viral factors contributing to CD8+ T cell failure in hepatitis C virus infection. *World J Gastroenterol* 2007; 13(36): 4839-4847

INTRODUCTION

The host immune response to pathogens involves various components of the immune system, including innate, humoral, and cellular immunity, the latter consisting of CD4+ and CD8+ T cells. All components of the immune response might have distinct roles in the outcome and pathogenesis of HCV infection and will be discussed in separate reviews in this issue of *WJG*. In this review, we will focus on the CD8+ T cell response to HCV infection. CD8+ T cells recognize viral antigen presented by HLA class I molecules on professional antigen presenting cells (CD8+ T cell priming) and on infected target cells (e.g. hepatocytes). Their antiviral activity includes cytotoxicity as well as the secretion of antiviral cytokines such as interferon-gamma (IFN- γ). In the following, successful virus-specific CD8+ T cell responses associated with viral clearance as well as ineffective CD8+ T cell responses present in persistent HCV infection will be described. The main focus of this review, however, is the multiple mechanisms that contribute to CD8+ T cell failure and viral persistence.

CD8+ T CELL RESPONSE IN ACUTE HCV INFECTION

During acute resolving HCV infection, vigorous virus-specific CD8+ T cell responses that target multiple epitopes can be detected approximately 4-8 wk after infection, and their emergence is temporally associated with the onset of liver disease^[1-4] (Figure 1A). However, the virus-specific CD8+ T cells are not able to secrete antiviral cytokines such as IFN- γ in this early phase of infection, a status referred to as 'stunned phenotype'^[2,3]. In a later phase of infection, virus-specific CD8+ T cells regain their ability to secrete antiviral cytokines, and this is temporally associated with a rapid decline of viremia and finally viral clearance. Knowledge about the intrahepatic virus-specific CD8+ T cell response during acute HCV infection was obtained from experimentally infected chimpanzees. Responses accumulate in the liver 8-14 wk after infection and coincide with liver disease as well as viral clearance^[5,6]. After resolution of infection, virus-specific CD4+ and

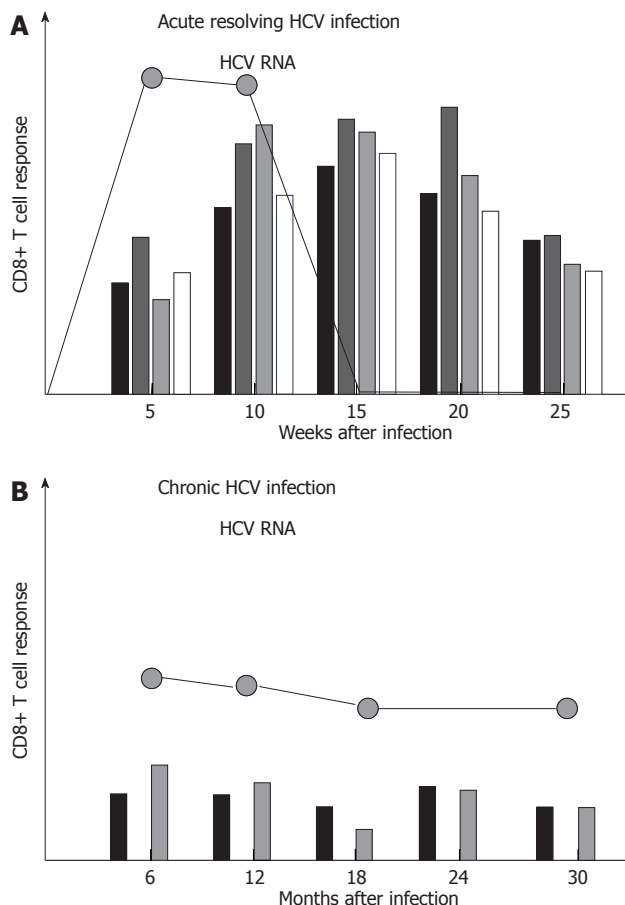


Figure 1 A: Virus-specific CD8+ T cell responses are strong, multi-specific, and sustained in acute resolving HCV infection; B: Virus-specific CD8+ T cell responses are weak and narrowly focused in chronic HCV infection.

CD8+ T cell responses persist for decades and can even outlast humoral responses^[7]. Virus-specific CD8+ T cells also play a role in mediating protective immunity. Indeed, evidence for protective immunity comes from both epidemiological studies as well as experimental studies^[8]. Chimpanzees re-challenged by HCV showed a shorter period and lower level of viremia than naïve animals^[9-11]. Sterilizing immunity against HCV, however, may not exist, since multiple episodes of heterologous or homologous re-infection have been observed in both, humans and chimpanzees.

In contrast to acute resolving HCV infection, the CD8+ T cell response in acute persisting HCV infection has been less defined. Previous reports comparing the CD8+ T cell response in acute resolving versus acute persisting HCV infection in chimpanzees and men found significantly weaker and more narrowly focused virus-specific CD8+ T cell responses in those subjects developing persistent infection^[1,2,5,6]. More recent studies, however, could not confirm this finding^[4,12,13]. For example, Cox *et al* performed a prospective longitudinal study in young iv drug users and analyzed the T cell response in 4 individuals with resolution of acute HCV infection and 15 individuals who progressed to chronic infection. Although all 4 individuals with resolving infection mounted virus-specific CD8+ T cell responses and those 4 individuals who lacked CD8+ T cell responses developed chronic infection, the

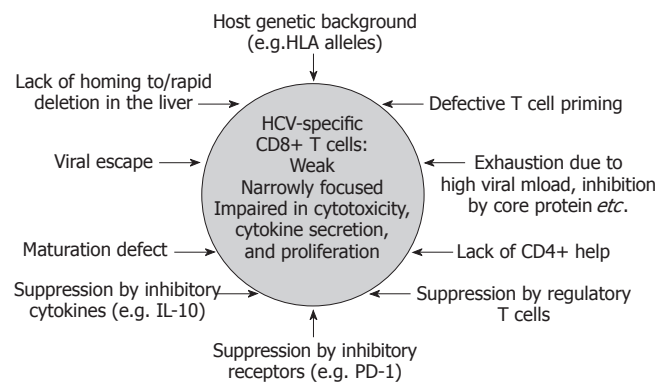


Figure 2 Possible Mechanisms of CD8+ T cell failure in persistent HCV infection.

CD8+ T cell response did not differ significantly between resolvers and persistently infected individuals^[4]. Urbani *et al* studied 6 patients with acute resolving and 11 patients with acute persisting HCV infection and found an association between strong and multispecific CD4+, but not CD8+ T cells with viral clearance. However, patients developing chronic infection displayed prolonged CD8+ T cell dysfunctions and maturational defects^[12]. This discordant role of CD4+ and CD8+ T cells was confirmed by Kaplan *et al*, albeit their analysis was limited to two HLA-A2 restricted CD8+ T cell epitopes^[13].

In sum, acute resolving HCV infection is associated with strong, broadly directed and sustained CD8+ T cell responses, while a universal picture of the CD8+ T cell response in acute persistent HCV infection has not yet been defined.

CD8 + T CELL RESPONSE IN CHRONIC HCV INFECTION

In contrast to acute resolving infection, CD8+ T cell responses are usually weak or even absent in chronic HCV infection, targeting only few epitopes^[14-22] (Figure 1B). In this context, it is important to point out that at least in some chronically infected patients, the CD8+ T cell response targets several epitopes^[17,18,22]. Importantly, however, these HCV-specific CD8+ T cells display functional impairments, including reduced cytotoxicity, reduced secretion of antiviral cytokines such as IFN- γ , and a reduced proliferative capacity^[23-25]. In addition, many CD8+ T cell responses do not target a present antigen, but rather a historical antigen due to viral escape (see below). Different mechanisms which might be involved in the failure of the HCV-specific CD8+ T cell response in persistent infection will be discussed in the following (Figure 2).

MECHANISMS OF CD8 + T CELL FAILURE

Primary failure and exhaustion

As discussed above, some patients with chronic HCV infection lack strong and multispecific CD8+ T cell responses, however, it is difficult to distinguish if virus-specific CD8+ T cell responses were not primed initially (primary CD8+ T cell failure) or responses were primed,

but vanished quickly (CD8+ T cell exhaustion). Results obtained from the early phase of acute HCV infection in chimpanzees and in health care workers infected through needle stick exposure support the hypothesis that CD8+ T cells are not primed at least in some patients with acute persisting HCV infection^[2,5,6]. In a prospective longitudinal study of young iv drug users, however, CD8+ T cell exhaustion was indeed demonstrated for at least one targeted epitope in each subject developing chronic infection^[4].

An impaired priming of HCV-specific CD8+ T cells might be mediated by numeric and functional impairments of antigen-presenting cells, e.g. macrophages and dendritic cells; however, this topic remains controversial^[26-35].

CD8+ T cell exhaustion might be explained by general as well as HCV-specific mechanisms. Of note, it has been demonstrated in the lymphocytic choriomeningitis virus (LCMV) mouse model that high viral loads lead to an unresponsive state of virus-specific CD8+ T cells, downregulation of T cell receptors, and finally physical deletion of virus-specific CD8+ T cells^[36-39]. Recent data indicate that the inhibitory receptor PD-1 might be involved in this process (see below), and it has been postulated that the detrimental effect of high viral load may not only apply in LCMV infection, but in different viral infections including HCV infection. Regarding HCV-specific mechanisms of CD8+ T cell exhaustion, the core protein has been reported to impair CD8+ T cell activation, e.g. through interaction with membrane-bound complement receptor gC1qR^[40-42].

Lack of CD4+ help

While CD8+ T cells are considered the major effector cells against viral pathogens, the successful elimination of HCV might be highly dependent on sufficient CD4+ T cell help. Indeed, it has been demonstrated in the LCMV mouse model that CD4+ T cell help is needed to sustain cytotoxic CD8+ T cell responses during chronic viral infections^[43]. In chronic HCV infection, however, CD4+ T cell responses are very weak or even absent and functionally impaired, e.g. secrete low amounts of IL-2^[44,45]. Findings in the chimpanzee model support the central role of CD4+ help in CD8+ T cell mediated viral clearance. When CD4+ T cells were depleted by neutralizing antibodies prior to viral re-challenge, HCV viremia was prolonged, CD8+ escape variants were selected and HCV finally persisted^[46]. Consistent with this concept, HCV-specific CD8+ T cell responses were seen almost exclusively in the face of a strong CD4+ T cell response in a study of acutely HCV infected patients^[1]. A recent study demonstrated that the outcome of acute HCV infection was associated with efficient virus-specific CD4+ T cell responses. In this study, however, HCV-specific CD8+ T cell responses were induced irrespective of virological outcome or HCV-specific CD4+ T cell responses^[13].

Suppression by regulatory T cells

In the last few years, the concept of regulatory T cells has undergone a comeback and different types of regulatory T cells have been characterized in different clinical settings. In HCV infection, the role of CD4+CD25+

Foxp3+ regulatory T cells as well as IL-10 producing CD8+ T cells has been defined. In chronically HCV infected patients, CD4+CD25+ T cells have been found in a higher frequency compared to individuals with resolved HCV infection or healthy controls^[47-49]. These regulatory T cells suppress the proliferation as well as interferon-gamma secretion of virus-specific CD8+ T cells *in vitro*. The suppression by CD4+CD25+ T cells was cell-cell contact dependent^[47,48], it was independent of suppressive cytokines such as IL-10 and TGF- β in some but not all studies^[47,48,50]. Interestingly, the suppression was not restricted to HCV-specific CD8+ T cells, but also included CD8+ T cells specific for other viruses, such as EBV or influenza^[47,50]. However, specificity *in vivo* might be mediated by the enrichment of CD4+CD25+ T cells in the liver^[51]. While CD4+CD25+ T cells might limit immunopathology in the chronic phase of HCV infection^[52], it has been suggested that they may facilitate viral persistence in the acute phase of infection. However, studies in larger cohorts of patients with acute HCV infection have not yet been reported. The induction of CD4+CD25+ regulatory T cells is still little characterized, however, they could be induced by certain HCV peptides from peripheral blood mononuclear cells (PBMCs) from HCV-infected, but not healthy individuals *in vitro*^[53].

Another type of regulatory T cells in HCV infection is virus-specific regulatory CD8+ T cells that express high levels of IL-10. These regulatory T cells have been detected in the liver of HCV-infected individuals; they could be expanded upon stimulation with HCV epitope peptides and their suppression of virus-specific CD8+ effector T cells could be blocked by neutralizing IL-10 antibodies^[54]. This virus-specific regulatory T cell population might have an important role in the prevention of liver damage during chronic HCV infection^[55].

The spectrum of regulatory T cells involved in HCV infection may further expand, since we recently described the induction of regulatory CD8+ T cells from the PBMC of HCV-infected patients which also expressed high levels of FoxP3 and CD25^[56]. A comprehensive review on the different types of regulatory T cells in HCV and HBV infection by Billerbeck *et al* is also included in this issue of *WJG*.

Inhibitory receptors: PD-1

The inhibitory receptor PD-1 ("programmed cell death 1") has been demonstrated to be a strong marker for exhausted virus-specific CD8+ T cells in the LCMV mouse model. The antibody-mediated blockade of the interaction between PD-1 and its ligand PD-L1 led to the restoration of cytokine secretion, proliferation, and cytotoxicity by the exhausted virus-specific CD8+ T cells and a substantial reduction in viral load^[57]. Similar roles of PD-1 have been shown in human chronic viral infections^[58] such as HIV^[59], HBV^[60,61], and HCV. In the acute phase of HCV infection, similar to LCMV infection, PD-1 is up-regulated on HCV-specific CD8+ T cells independent of outcome. However, in individuals with resolving infection PD-1 expression decreases soon, while in patients with a chronic course of infection, HCV-specific CD8+ T cells remain PD-1 positive^[62]. This finding is in parallel with

the “stunned” phenotype of HCV-specific CD8⁺ T cells in the early acute phase of infection, which is restored in resolving infection but remains in persisting infection^[2,3,24]. In chronic HCV infection, HCV-specific CD8⁺ T cells in the peripheral blood^[63] as well as liver^[64] have been shown to express high levels of PD-1. Blockade of PD-1/ PD-L1 interaction by antibodies restored cytokine production and proliferation of the exhausted CD8⁺ T cells from acute and chronic infection *in vitro*.

It is important to note, however, that the antibody-mediated blockade of the PD-1/PD-L1 pathway in chronically LCMV-infected mice did not result in viral clearance although a significant reduction of viral load was achieved. Even more importantly, PD-L1-/- mice died due to immunopathologic damage after infection with a LCMV strain usually establishing persistent infection^[57]. These findings indicate that a subtle balance in the blockade of the PD-1/PD-L1 pathway must be granted before it can be applied in the clinics.

Inhibitory cytokines: IL-10

Two recent reports on the role of IL-10 in the dysfunction of virus-specific T cells and viral persistence gained much attention in the field. These reports showed that in mice with persistent LCMV infection, IL-10 was highly up-regulated early in infection, which was associated with the dysfunction of virus-specific CD4⁺ and CD8⁺ T cells. The blockade of the IL-10/IL-10 receptor (IL-10R) pathway by a genetic approach or by an anti-IL-10R antibody early in infection, however, led to the restoration of T cell function and to clearance of infection^[65-67].

Although these reports definitely point towards an important general mechanism of T cell dysfunction, a role of IL-10 in HCV infection has been postulated before, and IL-10 therapy has even been tested in clinical trials in HCV infected patients. Indeed, many reports showed an association of IL-10 polymorphisms and outcome, disease progression, or response to antiviral therapy of HCV infection^[68-74], while other studies failed to confirm these data^[75-79]. Clinical trials with administration of recombinant IL-10 to patients with chronic HCV infection who had failed antiviral therapy with interferon-alpha led to a decrease in transaminases and histological disease progression; however, viral titers strongly increased in some IL-10 treated patients^[80,81]. This indicates that IL-10 might not only mediate viral dysfunction and thus facilitate viral persistence in acute infection, but may also reduce immunopathology in the chronic phase of infection. In this context, it is important to point out, however, that IL-10/IL-10R blockade in the LCMV mouse model did not result in severe immunopathology^[65,66].

The exact mechanism of HCV-induced up-regulation of IL-10 remains elusive. Some groups have reported induction of IL-10 production by monocytes^[82] or natural killer (NK) cells^[83] through core^[74,84], non-structural protein 3^[84], or 4^[82]. More intriguingly, HCV-specific CD8⁺ T cells with regulatory properties which produce IL-10 have been described in the liver of chronically HCV infected patients^[54]. These IL-10 producing intrahepatic CD8⁺ T cells were associated with mild inflammation and low progression of fibrosis in liver histology^[55], once more sug-

gesting that IL-10 may protect from immunopathology in chronic HCV infection. Blockade of the IL-10 pathway by anti-IL10R antibodies *in vitro* led to increased HCV-specific CD4⁺ T cell responses^[85]. In addition, antiviral therapy led to reduced production of IL-10 by virus-specific T cells in patients with chronic HCV infection^[86]. A direct inhibition of the IL-10 pathway, however, needs further careful evaluation in additional animal models before it can be transferred to men.

Viral escape

HCV is a RNA virus with an enormous replication rate (approximately 10¹² virions per day) with a RNA-dependent RNA polymerase that lacks a proofreading function. Therefore, multiple viral variants, called quasispecies, circulate in a single individual. It has been suggested that the selection of viral variants escaping from CD8⁺ T cell responses might facilitate the persistence of HCV infection. Indeed, the first evidence for viral escape in HCV infection came from chronically infected patients^[87] and experimentally infected chimpanzees^[88,89]. Chronically infected patients harbored variant viral sequences in targeted epitopes which were non-immunogenic and not cross-reactive with the prototype peptides. These viral escape mutations remained fixed over a follow-up time of up to four years, indicating that escape mutations occur early in infection^[87]. In the chimpanzee model, it could further be demonstrated that viral escape mutations occurred during the first 16 wk of infection and were associated with a chronic course of infection^[89].

Important additional information came from studies in acutely infected patients^[90-92] as well as population-based approaches^[90,93]. In these studies, viral escape from CD8⁺ T cell responses was demonstrated in patients developing persistent infection^[90-92], but not in individuals with resolving infection^[91,92]. Interestingly, many mutations outside of targeted CD8⁺ T cell epitopes represented conversion to consensus^[91], and the transmission of an HLA-B8 associated escape mutation to an HLA-B8 negative subject resulted in rapid reversion of the mutation^[90]. These results were supported by a study in a well-defined cohort of Irish women accidentally infected with HCV from a single source more than 20 years ago. In this unique cohort, amino acid substitutions in known epitopes were directed away from consensus in women having the HLA allele associated with that epitope, and toward consensus in those lacking the allele^[93]. These findings are in agreement with the concept of viral fitness cost, indicating that viral escape mutations are often associated with a reduced replicative capacity of the virus^[94]. In the absence of the T cell pressure, e.g. upon transmission to an individual negative for the restricting HLA allele, the virus reverts to consensus and thus regains its full replicative capacity. This phenomenon has been analyzed in more detail in the background of an immunodominant HLA-A2 restricted epitope, identifying that certain amino acid residue substitutions abolish HLA binding without strongly influencing viral replication, while some substitutions lead to a strong reduction of viral fitness^[95]. Importantly, there might be some CD8⁺ T cell epitopes which are not affected by viral escape

due to high functional constraints. For example, we have recently identified an HLA-A26 restricted epitope located at the NS5A/5B cleavage site which was targeted in all studied HLA-A26+ patients (3/3) with acute HCV infection and a significant number of patients with chronic HCV infection (3/15). However, the epitope sequence was highly conserved in HLA-A26 positive and negative patients, indicating that viral escape did not occur in this functionally constrained region^[96].

Based on the finding that immunodominant CD8+ T cell epitopes leave their footprint in viral sequences in chronic HCV infection^[90], viral genome sequencing studies were performed in order to identify footprints of additional potential CD8+ T cell epitopes^[97,98]. In addition to previously defined epitopes, these studies identified HLA allele dependent polymorphisms and thus candidate CD8+ T cell epitopes. Importantly, the strongest association with any HLA allele in the study by Timm *et al* was found for HLA-B27 in a region that was shown to contain an immunodominant HLA-B27 restricted CD8+ T cell epitope by an independent study in another patient cohort^[99].

There are different molecular mechanisms by which a certain mutation escapes from the CD8+ T cell response. Especially those mutations located at the HLA binding anchors, usually P2 and the C-terminal amino acid, lead to the interruption of the peptide binding to the HLA molecule. Mutations in the center of the epitope, in contrast, are more likely to interfere with T cell receptor (TCR) recognition^[95]. Mutations in the flanking region, however, prevent proteasomal epitope processing^[90,100,101].

The determinants of viral escape are less understood. In the chimpanzee model of HCV, it has been shown that upon depletion of CD4+ T cells in the acute phase of infection viral escape from the CD8+ T cell response occurs and is associated with a persistent course of infection^[46]. This finding has led to the hypothesis that viral escape is caused by insufficient CD4+ help. Other studies indicate that a limited T cell receptor (TCR) diversity might be responsible for viral escape^[102]. Of note, viral escape does not occur in the context of dysfunctional CD8+ T cell responses^[103]. The strong association between HLA-B27 and viral escape within an immunodominant HLA-B27 restricted epitope as well as the suggestion that escape variant epitopes might preferentially be restricted by HLA-B alleles indicates that the restricting HLA allele background also plays an important role in determining viral escape^[97-99].

Lack of homing to the liver

Experimentally HCV infected chimpanzees which progressed to viral persistence without temporary viral control lacked virus-specific CD8+ T cell responses in the liver despite of detectable responses in the peripheral blood^[2]. This finding led to the tempting hypothesis that the failure of the virus-specific CD8+ T cell response might be caused by an insufficient homing to the primary location of infection, the liver. However, in chronically HCV infected patients virus-specific CD8+ T cells are detectable and even enriched in the liver^[22,25,104-108]. In a comprehensive study comparing the overall breadth and

vigor of CD8+ T cell responses in the peripheral blood and liver of chronically HCV infected patients, we found that virus-specific CD8+ T cell responses were strongly enriched in the liver. Many responses were only detectable in the liver; however, few responses were limited to the peripheral blood (Neumann-Haefelin *et al*, unpublished results). Therefore, it is possible that a defective homing of HCV-specific CD8+ T cells or their rapid deletion in the liver also contributes to T cell failure and viral persistence in a subset of patients.

ROLE OF THE HOST HLA CLASS I BACKGROUND

CD8+ T cells recognize antigens presented by human leukocyte antigen (HLA) class I molecules. It has, therefore, been suggested that different HLA class I alleles are associated with differential outcomes of HCV infection, e.g. viral clearance versus persistence^[109]. Analysis of the role of HLA alleles in viral infections are hindered by multiple factors, including the wide polymorphism of HLA alleles, their association with other genetic characteristics e.g. in certain racial backgrounds (founder effect)^[110], and the variability of viral strains (genotypes, quasispecies *etc.*). However, an Irish cohort of women accidentally infected with HCV (genotype 1b) from a single source more than 20 years ago, represents a homogeneous group in which the role of HLA alleles in the outcome of HCV infection could be studied^[111]. Importantly, the HLA class I alleles A3, B27 and Cw*01 were significantly associated with viral clearance, while B8 was associated with viral persistence. Interestingly, the strongest protective effect was observed for HLA-B27: 80% (12/15) of B27 positive women were able to clear the infection spontaneously, while only a minority developed chronic infection. We recently identified an immunodominant HLA-B27 restricted HCV-specific CD8+ T cell epitope, which was targeted in the vast majority (5/6) of B27 positive Irish women who had cleared the infection^[99]. Of note, such a clear dominance of a single epitope-specific CD8+ T cell response has not been described for any other HLA allele in HCV infection. In chronically infected patients, still a remarkable percentage of patients (3/8) recognized the epitope. However, most B27 positive chronically infected patients had evidence of viral escape within the otherwise conserved viral region containing this epitope. Thus, a single immunodominant HLA-B27 restricted CD8+ T cell epitopes might mediate both, clearance of HCV infection in the majority of B27 positive individuals, and viral evolution associated with viral persistence in a minority of individuals.

Strikingly, a very similar frequency of viral escape variation was demonstrated within an immunodominant HLA-B8 restricted CD8+ T cell epitopes^[90]. This indicates that in the background of both, a protective HLA allele (B27) as well as a detrimental HLA allele (B8) the principle mechanisms of CD8+ T cell failure might be the same. More precise details such as viral fitness cost associated with the respective escape variation^[94,95], T cell receptor (TCR) diversity^[102] or heterologous immunity^[112,113]

may play an additional critical role in the definition of a protective HLA allele.

Two other population studies in more heterogeneous cohorts showed an association between HLA-B57 and HCV clearance in Caucasian as well as African Americans and West Africans^[114,115]. Interestingly, HLA-B27 and HLA-B57 have also been shown to be protective in HIV infection, being strongly associated with low viral titers, low CD4+ T cell decline, and long-term non-progression of the disease^[116]. Thus, a picture emerges that the same HLA alleles may confer protection in different clinical infections, indicating that similar mechanisms of viral control and disease progression apply in these infections. A better understanding of the host-virus interactions leading to different clinical outcomes of HCV infection will be important not only to understand the mechanisms of viral clearance and persistence, but also for the development of new antiviral vaccine strategies.

CONCLUSION

CD8+ T cells are generally thought to be the major effectors in viral infections; however, multiple host and viral mechanisms contribute to the failure of antiviral CD8+ T cell responses and viral persistence in the majority of HCV infected patients. In the last few years compelling progress has been achieved in the understanding of these mechanisms (compare with^[117]). These findings are not only important for the development of successful immunoprophylactic approaches, but may also be more directly adopted for immunotherapeutic interventions.

REFERENCES

- Grüner NH, Gerlach TJ, Jung MC, Diepolder HM, Schirren CA, Schraut WW, Hoffmann R, Zachoval R, Santantonio T, Cucchiari M, Cerny A, Pape GR. Association of hepatitis C virus-specific CD8+ T cells with viral clearance in acute hepatitis C. *J Infect Dis* 2000; **181**: 1528-1536
- Thimme R, Oldach D, Chang KM, Steiger C, Ray SC, Chisari FV. Determinants of viral clearance and persistence during acute hepatitis C virus infection. *J Exp Med* 2001; **194**: 1395-1406
- Lechner F, Wong DK, Dunbar PR, Chapman R, Chung RT, Dohrenwend P, Robbins G, Phillips R, Klenerman P, Walker BD. Analysis of successful immune responses in persons infected with hepatitis C virus. *J Exp Med* 2000; **191**: 1499-1512
- Cox AL, Mosbruger T, Lauer GM, Pardoll D, Thomas DL, Ray SC. Comprehensive analyses of CD8+ T cell responses during longitudinal study of acute human hepatitis C. *Hepatology* 2005; **42**: 104-112
- Thimme R, Bukh J, Spangenberg HC, Wieland S, Pemberton J, Steiger C, Govindarajan S, Purcell RH, Chisari FV. Viral and immunological determinants of hepatitis C virus clearance, persistence, and disease. *Proc Natl Acad Sci USA* 2002; **99**: 15661-15668
- Cooper S, Erickson AL, Adams EJ, Kansopon J, Weiner AJ, Chien DY, Houghton M, Parham P, Walker CM. Analysis of a successful immune response against hepatitis C virus. *Immunity* 1999; **10**: 439-449
- Takaki A, Wiese M, Maertens G, Depla E, Seifert U, Liebetrau A, Miller JL, Manns MP, Rehermann B. Cellular immune responses persist and humoral responses decrease two decades after recovery from a single-source outbreak of hepatitis C. *Nat Med* 2000; **6**: 578-582
- Mehta SH, Cox A, Hoover DR, Wang XH, Mao Q, Ray S, Strathdee SA, Vlahov D, Thomas DL. Protection against persistence of hepatitis C. *Lancet* 2002; **359**: 1478-1483
- Bassett SE, Guerra B, Brasky K, Miskovsky E, Houghton M, Klimpel GR, Lanford RE. Protective immune response to hepatitis C virus in chimpanzees rechallenged following clearance of primary infection. *Hepatology* 2001; **33**: 1479-1487
- Weiner AJ, Paliard X, Selby MJ, Medina-Selby A, Coit D, Nguyen S, Kansopon J, Arian CL, Ng P, Tucker J, Lee CT, Polakos NK, Han J, Wong S, Lu HH, Rosenberg S, Brasky KM, Chien D, Kuo G, Houghton M. Intrahepatic genetic inoculation of hepatitis C virus RNA confers cross-protective immunity. *J Virol* 2001; **75**: 7142-7148
- Major ME, Mihalik K, Puig M, Rehermann B, Nascimbeni M, Rice CM, Feinstone SM. Previously infected and recovered chimpanzees exhibit rapid responses that control hepatitis C virus replication upon rechallenge. *J Virol* 2002; **76**: 6586-6595
- Urbani S, Amadei B, Fiscaro P, Tola D, Orlandini A, Sacchelli L, Mori C, Missale G, Ferrari C. Outcome of acute hepatitis C is related to virus-specific CD4 function and maturation of antiviral memory CD8 responses. *Hepatology* 2006; **44**: 126-139
- Kaplan DE, Sugimoto K, Newton K, Valiga ME, Ikeda F, Aytaman A, Nunes FA, Lucey MR, Vance BA, Vonderheide RH, Reddy KR, McKeating JA, Chang KM. Discordant role of CD4 T-cell response relative to neutralizing antibody and CD8 T-cell responses in acute hepatitis C. *Gastroenterology* 2007; **132**: 654-666
- Battegay M, Fikes J, Di Bisceglie AM, Wentworth PA, Sette A, Celis E, Ching WM, Grakoui A, Rice CM, Kurokohchi K. Patients with chronic hepatitis C have circulating cytotoxic T cells which recognize hepatitis C virus-encoded peptides binding to HLA-A2.1 molecules. *J Virol* 1995; **69**: 2462-2470
- Cerny A, McHutchison JG, Pasquinelli C, Brown ME, Brothers MA, Grabscheid B, Fowler P, Houghton M, Chisari FV. Cytotoxic T lymphocyte response to hepatitis C virus-derived peptides containing the HLA A2.1 binding motif. *J Clin Invest* 1995; **95**: 521-530
- Chang KM, Thimme R, Melpolder JJ, Oldach D, Pemberton J, Moorhead-Loudis J, McHutchison JG, Alter HJ, Chisari FV. Differential CD4(+) and CD8(+) T-cell responsiveness in hepatitis C virus infection. *Hepatology* 2001; **33**: 267-276
- Lauer GM, Barnes E, Lucas M, Timm J, Ouchi K, Kim AY, Day CL, Robbins GK, Casson DR, Reiser M, Dusheiko G, Allen TM, Chung RT, Walker BD, Klenerman P. High resolution analysis of cellular immune responses in resolved and persistent hepatitis C virus infection. *Gastroenterology* 2004; **127**: 924-936
- Lauer GM, Ouchi K, Chung RT, Nguyen TN, Day CL, Purkis DR, Reiser M, Kim AY, Lucas M, Klenerman P, Walker BD. Comprehensive analysis of CD8(+)-T-cell responses against hepatitis C virus reveals multiple unpredicted specificities. *J Virol* 2002; **76**: 6104-6113
- Rehermann B, Chang KM, McHutchison JG, Kokka R, Houghton M, Chisari FV. Quantitative analysis of the peripheral blood cytotoxic T lymphocyte response in patients with chronic hepatitis C virus infection. *J Clin Invest* 1996; **98**: 1432-1440
- Wong DK, Dudley DD, Dohrenwend PB, Lauer GM, Chung RT, Thomas DL, Walker BD. Detection of diverse hepatitis C virus (HCV)-specific cytotoxic T lymphocytes in peripheral blood of infected persons by screening for responses to all translated proteins of HCV. *J Virol* 2001; **75**: 1229-1235
- Koziel MJ, Dudley D, Afdhal N, Choo QL, Houghton M, Ralston R, Walker BD. Hepatitis C virus (HCV)-specific cytotoxic T lymphocytes recognize epitopes in the core and envelope proteins of HCV. *J Virol* 1993; **67**: 7522-7532
- Koziel MJ, Dudley D, Afdhal N, Grakoui A, Rice CM, Choo QL, Houghton M, Walker BD. HLA class I-restricted cytotoxic T lymphocytes specific for hepatitis C virus. Identification of multiple epitopes and characterization of patterns of cytokine release. *J Clin Invest* 1995; **96**: 2311-2321
- Wedemeyer H, He XS, Nascimbeni M, Davis AR, Greenberg HB, Hoofnagle JH, Liang TJ, Alter H, Rehermann B. Impaired effector function of hepatitis C virus-specific CD8+ T cells in chronic hepatitis C virus infection. *J Immunol* 2002; **169**:

- 3447-3458
- 24 **Urbani S**, Boni C, Missale G, Elia G, Cavallo C, Massari M, Raimondo G, Ferrari C. Virus-specific CD8+ lymphocytes share the same effector-memory phenotype but exhibit functional differences in acute hepatitis B and C. *J Virol* 2002; **76**: 12423-12434
 - 25 **Spangenberg HC**, Viazov S, Kersting N, Neumann-Haefelin C, McKinney D, Roggendorf M, von Weizsäcker F, Blum HE, Thimme R. Intrahepatic CD8+ T-cell failure during chronic hepatitis C virus infection. *Hepatology* 2005; **42**: 828-837
 - 26 **Bain C**, Fatmi A, Zoulim F, Zarski JP, Trépo C, Inchauspé G. Impaired allostimulatory function of dendritic cells in chronic hepatitis C infection. *Gastroenterology* 2001; **120**: 512-524
 - 27 **Lee CH**, Choi YH, Yang SH, Lee CW, Ha SJ, Sung YC. Hepatitis C virus core protein inhibits interleukin 12 and nitric oxide production from activated macrophages. *Virology* 2001; **279**: 271-279
 - 28 **Sarobe P**, Lasarte JJ, Casares N, López-Díaz de Cerio A, Baixeras E, Labarga P, García N, Borrás-Cuesta F, Prieto J. Abnormal priming of CD4(+) T cells by dendritic cells expressing hepatitis C virus core and E1 proteins. *J Virol* 2002; **76**: 5062-5070
 - 29 **Anthony DD**, Yonkers NL, Post AB, Asaad R, Heinzel FP, Lederman MM, Lehmann PV, Valdez H. Selective impairments in dendritic cell-associated function distinguish hepatitis C virus and HIV infection. *J Immunol* 2004; **172**: 4907-4916
 - 30 **Kanto T**, Hayashi N, Takehara T, Tatsumi T, Kuzushita N, Ito A, Sasaki Y, Kasahara A, Hori M. Impaired allostimulatory capacity of peripheral blood dendritic cells recovered from hepatitis C virus-infected individuals. *J Immunol* 1999; **162**: 5584-5591
 - 31 **Della Bella S**, Crosignani A, Riva A, Presicce P, Benetti A, Longhi R, Podda M, Villa ML. Decrease and dysfunction of dendritic cells correlate with impaired hepatitis C virus-specific CD4+ T-cell proliferation in patients with hepatitis C virus infection. *Immunology* 2007; **121**: 283-292
 - 32 **Longman RS**, Talal AH, Jacobson IM, Albert ML, Rice CM. Presence of functional dendritic cells in patients chronically infected with hepatitis C virus. *Blood* 2004; **103**: 1026-1029
 - 33 **Rollier C**, Drexhage JA, Verstrepen BE, Verschoor EJ, Bontrop RE, Koopman G, Heeney JL. Chronic hepatitis C virus infection established and maintained in chimpanzees independent of dendritic cell impairment. *Hepatology* 2003; **38**: 851-858
 - 34 **Larsson M**, Babcock E, Grakoui A, Shoukry N, Lauer G, Rice C, Walker C, Bhardwaj N. Lack of phenotypic and functional impairment in dendritic cells from chimpanzees chronically infected with hepatitis C virus. *J Virol* 2004; **78**: 6151-6161
 - 35 **Piccioli D**, Tavarini S, Nuti S, Colombatto P, Brunetto M, Bonino F, Ciccorossi P, Zorat F, Pozzato G, Comar C, Abrignani S, Wack A. Comparable functions of plasmacytoid and monocyte-derived dendritic cells in chronic hepatitis C patients and healthy donors. *J Hepatol* 2005; **42**: 61-67
 - 36 **Moskophidis D**, Lechner F, Pircher H, Zinkernagel RM. Virus persistence in acutely infected immunocompetent mice by exhaustion of antiviral cytotoxic effector T cells. *Nature* 1993; **362**: 758-761
 - 37 **Gallimore A**, Glithero A, Godkin A, Tissot AC, Plückthun A, Elliott T, Hengartner H, Zinkernagel R. Induction and exhaustion of lymphocytic choriomeningitis virus-specific cytotoxic T lymphocytes visualized using soluble tetrameric major histocompatibility complex class I-peptide complexes. *J Exp Med* 1998; **187**: 1383-1393
 - 38 **Ou R**, Zhou S, Huang L, Moskophidis D. Critical role for alpha/beta and gamma interferons in persistence of lymphocytic choriomeningitis virus by clonal exhaustion of cytotoxic T cells. *J Virol* 2001; **75**: 8407-8423
 - 39 **Zajac AJ**, Blattman JN, Murali-Krishna K, Sourdive DJ, Suresh M, Altman JD, Ahmed R. Viral immune evasion due to persistence of activated T cells without effector function. *J Exp Med* 1998; **188**: 2205-2213
 - 40 **Kittlesen DJ**, Chianese-Bullock KA, Yao ZQ, Braciale TJ, Hahn YS. Interaction between complement receptor gC1qR and hepatitis C virus core protein inhibits T-lymphocyte proliferation. *J Clin Invest* 2000; **106**: 1239-1249
 - 41 **Yao ZQ**, Nguyen DT, Hiotellis AI, Hahn YS. Hepatitis C virus core protein inhibits human T lymphocyte responses by a complement-dependent regulatory pathway. *J Immunol* 2001; **167**: 5264-5272
 - 42 **Yao ZQ**, Shata MT, Tricoche N, Shan MM, Brotman B, Pfahler W, Hahn YS, Prince AM. gC1qR expression in chimpanzees with resolved and chronic infection: potential role of HCV core/gC1qR-mediated T cell suppression in the outcome of HCV infection. *Virology* 2006; **346**: 324-337
 - 43 **Matloubian M**, Concepcion RJ, Ahmed R. CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection. *J Virol* 1994; **68**: 8056-8063
 - 44 **Day CL**, Seth NP, Lucas M, Appel H, Gauthier L, Lauer GM, Robbins GK, Szczepiorkowski ZM, Casson DR, Chung RT, Bell S, Harcourt G, Walker BD, Klenerman P, Wucherpfennig KW. Ex vivo analysis of human memory CD4 T cells specific for hepatitis C virus using MHC class II tetramers. *J Clin Invest* 2003; **112**: 831-842
 - 45 **Semmo N**, Day CL, Ward SM, Lucas M, Harcourt G, Loughry A, Klenerman P. Preferential loss of IL-2-secreting CD4+ T helper cells in chronic HCV infection. *Hepatology* 2005; **41**: 1019-1028
 - 46 **Grakoui A**, Shoukry NH, Woollard DJ, Han JH, Hanson HL, Ghayeb J, Murthy KK, Rice CM, Walker CM. HCV persistence and immune evasion in the absence of memory T cell help. *Science* 2003; **302**: 659-662
 - 47 **Boettler T**, Spangenberg HC, Neumann-Haefelin C, Panther E, Urbani S, Ferrari C, Blum HE, von Weizsäcker F, Thimme R. T cells with a CD4+CD25+ regulatory phenotype suppress in vitro proliferation of virus-specific CD8+ T cells during chronic hepatitis C virus infection. *J Virol* 2005; **79**: 7860-7867
 - 48 **Cabrera R**, Tu Z, Xu Y, Firpi RJ, Rosen HR, Liu C, Nelson DR. An immunomodulatory role for CD4(+)CD25(+) regulatory T lymphocytes in hepatitis C virus infection. *Hepatology* 2004; **40**: 1062-1071
 - 49 **Sugimoto K**, Ikeda F, Stadanlick J, Nunes FA, Alter HJ, Chang KM. Suppression of HCV-specific T cells without differential hierarchy demonstrated ex vivo in persistent HCV infection. *Hepatology* 2003; **38**: 1437-1448
 - 50 **Rushbrook SM**, Ward SM, Unitt E, Vowler SL, Lucas M, Klenerman P, Alexander GJ. Regulatory T cells suppress in vitro proliferation of virus-specific CD8+ T cells during persistent hepatitis C virus infection. *J Virol* 2005; **79**: 7852-7859
 - 51 **Ward SM**, Fox BC, Brown PJ, Worthington J, Fox SB, Chapman RW, Fleming KA, Banham AH, Klenerman P. Quantification and localisation of FOXP3+ T lymphocytes and relation to hepatic inflammation during chronic HCV infection. *J Hepatol* 2007; **47**: 316-324
 - 52 **Bolacchi F**, Sinistro A, Ciaprini C, Demin F, Capozzi M, Carducci FC, Drapeau CM, Rocchi G, Bergamini A. Increased hepatitis C virus (HCV)-specific CD4+CD25+ regulatory T lymphocytes and reduced HCV-specific CD4+ T cell response in HCV-infected patients with normal versus abnormal alanine aminotransferase levels. *Clin Exp Immunol* 2006; **144**: 188-196
 - 53 **Li S**, Jones KL, Woollard DJ, Dromey J, Paukovics G, Plebanski M, Gowans EJ. Defining target antigens for CD25+ FOXP3+ IFN-gamma- regulatory T cells in chronic hepatitis C virus infection. *Immunol Cell Biol* 2007; **85**: 197-204
 - 54 **Accapezzato D**, Francavilla V, Paroli M, Casciaro M, Chircu LV, Cividini A, Abrignani S, Mondelli MU, Barnaba V. Hepatic expansion of a virus-specific regulatory CD8(+) T cell population in chronic hepatitis C virus infection. *J Clin Invest* 2004; **113**: 963-972
 - 55 **Abel M**, Sène D, Pol S, Bourlière M, Poynard T, Charlotte F, Cacoub P, Caillat-Zucman S. Intrahepatic virus-specific IL-10-producing CD8 T cells prevent liver damage during chronic hepatitis C virus infection. *Hepatology* 2006; **44**: 1607-1616
 - 56 **Billerbeck E**, Blum HE, Thimme R. Parallel expansion of human virus-specific FoxP3- effector memory and de novo-generated FoxP3+ regulatory CD8+ T cells upon antigen

- recognition in vitro. *J Immunol* 2007; **179**: 1039-1048
- 57 **Barber DL**, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe AH, Freeman GJ, Ahmed R. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* 2006; **439**: 682-687
 - 58 **Sharpe AH**, Wherry EJ, Ahmed R, Freeman GJ. The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. *Nat Immunol* 2007; **8**: 239-245
 - 59 **Day CL**, Kaufmann DE, Kiepiela P, Brown JA, Moodley ES, Reddy S, Mackey EW, Miller JD, Leslie AJ, DePierres C, Mncube Z, Duraiswamy J, Zhu B, Eichbaum Q, Altfeld M, Wherry EJ, Coovadia HM, Goulder PJ, Klennerman P, Ahmed R, Freeman GJ, Walker BD. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* 2006; **443**: 350-354
 - 60 **Isogawa M**, Furuichi Y, Chisari FV. Oscillating CD8(+) T cell effector functions after antigen recognition in the liver. *Immunity* 2005; **23**: 53-63
 - 61 **Boettler T**, Panther E, Bengsch B, Nazarova N, Spangenberg HC, Blum HE, Thimme R. Expression of the interleukin-7 receptor alpha chain (CD127) on virus-specific CD8+ T cells identifies functionally and phenotypically defined memory T cells during acute resolving hepatitis B virus infection. *J Virol* 2006; **80**: 3532-3540
 - 62 **Urbani S**, Amadei B, Tola D, Massari M, Schivazappa S, Missale G, Ferrari C. PD-1 expression in acute hepatitis C virus (HCV) infection is associated with HCV-specific CD8 exhaustion. *J Virol* 2006; **80**: 11398-11403
 - 63 **Penna A**, Pilli M, Zerbin A, Orlandini A, Mezzadri S, Sacchelli L, Missale G, Ferrari C. Dysfunction and functional restoration of HCV-specific CD8 responses in chronic hepatitis C virus infection. *Hepatology* 2007; **45**: 588-601
 - 64 **Radziejewicz H**, Ibegbu CC, Fernandez ML, Workowski KA, Obideen K, Wehbi M, Hanson HL, Steinberg JP, Masopust D, Wherry EJ, Altman JD, Rouse BT, Freeman GJ, Ahmed R, Grakoui A. Liver-infiltrating lymphocytes in chronic human hepatitis C virus infection display an exhausted phenotype with high levels of PD-1 and low levels of CD127 expression. *J Virol* 2007; **81**: 2545-2553
 - 65 **Brooks DG**, Trifilo MJ, Edelmann KH, Teyton L, McGavern DB, Oldstone MB. Interleukin-10 determines viral clearance or persistence in vivo. *Nat Med* 2006; **12**: 1301-1309
 - 66 **Ejrnaes M**, Filippi CM, Martinic MM, Ling EM, Togher LM, Crotty S, von Herrath MG. Resolution of a chronic viral infection after interleukin-10 receptor blockade. *J Exp Med* 2006; **203**: 2461-2472
 - 67 **Blackburn SD**, Wherry EJ. IL-10, T cell exhaustion and viral persistence. *Trends Microbiol* 2007; **15**: 143-146
 - 68 **Yee LJ**, Tang J, Gibson AW, Kimberly R, Van Leeuwen DJ, Kaslow RA. Interleukin 10 polymorphisms as predictors of sustained response in antiviral therapy for chronic hepatitis C infection. *Hepatology* 2001; **33**: 708-712
 - 69 **Lio D**, Caruso C, Di Stefano R, Colonna Romano G, Ferraro D, Scola L, Crivello A, Licata A, Valenza LM, Candore G, Craxi A, Almasio PL. IL-10 and TNF-alpha polymorphisms and the recovery from HCV infection. *Hum Immunol* 2003; **64**: 674-680
 - 70 **Knapp S**, Hennig BJ, Frodsham AJ, Zhang L, Hellier S, Wright M, Goldin R, Hill AV, Thomas HC, Thursz MR. Interleukin-10 promoter polymorphisms and the outcome of hepatitis C virus infection. *Immunogenetics* 2003; **55**: 362-369
 - 71 **Mangia A**, Santoro R, Piattelli M, Paziienza V, Grifa G, Iacobellis A, Andriulli A. IL-10 haplotypes as possible predictors of spontaneous clearance of HCV infection. *Cytokine* 2004; **25**: 103-109
 - 72 **Oleksyk TK**, Thio CL, Truelove AL, Goedert JJ, Donfield SM, Kirk GD, Thomas DL, O'Brien SJ, Smith MW. Single nucleotide polymorphisms and haplotypes in the IL10 region associated with HCV clearance. *Genes Immun* 2005; **6**: 347-357
 - 73 **Paladino N**, Fainboim H, Theiler G, Schroder T, Muñoz AE, Flores AC, Galdame O, Fainboim L. Gender susceptibility to chronic hepatitis C virus infection associated with interleukin 10 promoter polymorphism. *J Virol* 2006; **80**: 9144-9150
 - 74 **Aborsangaya KB**, Dembinski I, Khatkar S, Alphonse MP, Nickerson P, Rempel JD. Impact of aboriginal ethnicity on HCV core-induced IL-10 synthesis: interaction with IL-10 gene polymorphisms. *Hepatology* 2007; **45**: 623-630
 - 75 **Constantini PK**, Wawrzynowicz-Syczewska M, Clare M, Boron-Kaczmarek A, McFarlane IG, Cramp ME, Donaldson PT. Interleukin-1, interleukin-10 and tumour necrosis factor-alpha gene polymorphisms in hepatitis C virus infection: an investigation of the relationships with spontaneous viral clearance and response to alpha-interferon therapy. *Liver* 2002; **22**: 404-412
 - 76 **Barrett S**, Collins M, Kenny C, Ryan E, Keane CO, Crowe J. Polymorphisms in tumour necrosis factor-alpha, transforming growth factor-beta, interleukin-10, interleukin-6, interferon-gamma, and outcome of hepatitis C virus infection. *J Med Virol* 2003; **71**: 212-218
 - 77 **Minton EJ**, Smillie D, Smith P, Shipley S, McKendrick MW, Gleeson DC, Underwood JC, Cannings C, Wilson AG. Clearance of hepatitis C virus is not associated with single nucleotide polymorphisms in the IL-1, -6, or -10 genes. *Hum Immunol* 2005; **66**: 127-132
 - 78 **Abbott WG**, Rigopoulou E, Haigh P, Cooksley H, Mullerova I, Novelli M, Winstanley A, Williams R, Naoumov NV. Single nucleotide polymorphisms in the interferon-gamma and interleukin-10 genes do not influence chronic hepatitis C severity or T-cell reactivity to hepatitis C virus. *Liver Int* 2004; **24**: 90-97
 - 79 **Kusumoto K**, Uto H, Hayashi K, Takahama Y, Nakao H, Suruki R, Stuver SO, Ido A, Tsubouchi H. Interleukin-10 or tumor necrosis factor-alpha polymorphisms and the natural course of hepatitis C virus infection in a hyperendemic area of Japan. *Cytokine* 2006; **34**: 24-31
 - 80 **Nelson DR**, Lauwers GY, Lau JY, Davis GL. Interleukin 10 treatment reduces fibrosis in patients with chronic hepatitis C: a pilot trial of interferon nonresponders. *Gastroenterology* 2000; **118**: 655-660
 - 81 **Nelson DR**, Tu Z, Soldevila-Pico C, Abdelmalek M, Zhu H, Xu YL, Cabrera R, Liu C, Davis GL. Long-term interleukin 10 therapy in chronic hepatitis C patients has a proviral and anti-inflammatory effect. *Hepatology* 2003; **38**: 859-868
 - 82 **Brady MT**, MacDonald AJ, Rowan AG, Mills KH. Hepatitis C virus non-structural protein 4 suppresses Th1 responses by stimulating IL-10 production from monocytes. *Eur J Immunol* 2003; **33**: 3448-3457
 - 83 **De Maria A**, Fogli M, Mazza S, Basso M, Picciotto A, Costa P, Congia S, Mingari MC, Moretta L. Increased natural cytotoxicity receptor expression and relevant IL-10 production in NK cells from chronically infected viremic HCV patients. *Eur J Immunol* 2007; **37**: 445-455
 - 84 **Dolganiuc A**, Kodys K, Kopasz A, Marshall C, Do T, Romics L, Mandrekar P, Zapp M, Szabo G. Hepatitis C virus core and nonstructural protein 3 proteins induce pro- and anti-inflammatory cytokines and inhibit dendritic cell differentiation. *J Immunol* 2003; **170**: 5615-5624
 - 85 **Rigopoulou EI**, Abbott WG, Haigh P, Naoumov NV. Blocking of interleukin-10 receptor--a novel approach to stimulate T-helper cell type 1 responses to hepatitis C virus. *Clin Immunol* 2005; **117**: 57-64
 - 86 **Cramp ME**, Rossol S, Chokshi S, Carucci P, Williams R, Naoumov NV. Hepatitis C virus-specific T-cell reactivity during interferon and ribavirin treatment in chronic hepatitis C. *Gastroenterology* 2000; **118**: 346-355
 - 87 **Chang KM**, Rehmann B, McHutchison JG, Pasquinelli C, Southwood S, Sette A, Chisari FV. Immunological significance of cytotoxic T lymphocyte epitope variants in patients chronically infected by the hepatitis C virus. *J Clin Invest* 1997; **100**: 2376-2385
 - 88 **Weiner A**, Erickson AL, Kansopon J, Crawford K, Muchmore E, Hughes AL, Houghton M, Walker CM. Persistent hepatitis C virus infection in a chimpanzee is associated with emergence of a cytotoxic T lymphocyte escape variant. *Proc Natl Acad Sci USA* 1995; **92**: 2755-2759
 - 89 **Erickson AL**, Kimura Y, Igarashi S, Eichelberger J, Houghton M, Sidney J, McKinney D, Sette A, Hughes AL, Walker

- CM. The outcome of hepatitis C virus infection is predicted by escape mutations in epitopes targeted by cytotoxic T lymphocytes. *Immunity* 2001; **15**: 883-895
- 90 **Timm J**, Lauer GM, Kavanagh DG, Sheridan I, Kim AY, Lucas M, Pillay T, Ouchi K, Reyor LL, Schulze zur Wiesch J, Gandhi RT, Chung RT, Bhardwaj N, Klennerman P, Walker BD, Allen TM. CD8 epitope escape and reversion in acute HCV infection. *J Exp Med* 2004; **200**: 1593-1604
 - 91 **Cox AL**, Mosbruger T, Mao Q, Liu Z, Wang XH, Yang HC, Sidney J, Sette A, Pardoll D, Thomas DL, Ray SC. Cellular immune selection with hepatitis C virus persistence in humans. *J Exp Med* 2005; **201**: 1741-1752
 - 92 **Tester I**, Smyk-Pearson S, Wang P, Wertheimer A, Yao E, Lewinsohn DM, Tavis JE, Rosen HR. Immune evasion versus recovery after acute hepatitis C virus infection from a shared source. *J Exp Med* 2005; **201**: 1725-1731
 - 93 **Ray SC**, Fanning L, Wang XH, Netski DM, Kenny-Walsh E, Thomas DL. Divergent and convergent evolution after a common-source outbreak of hepatitis C virus. *J Exp Med* 2005; **201**: 1753-1759
 - 94 **Altman JD**, Feinberg MB. HIV escape: there and back again. *Nat Med* 2004; **10**: 229-230
 - 95 **Söderholm J**, Ahlén G, Kaul A, Frelín L, Alheim M, Barnfield C, Liljeström P, Weiland O, Milich DR, Bartenschlager R, Sällberg M. Relation between viral fitness and immune escape within the hepatitis C virus protease. *Gut* 2006; **55**: 266-274
 - 96 **Neumann-Haefelin C**, Killinger T, Timm J, Southwood S, McKinney D, Blum HE, Thimme R. Absence of viral escape within a frequently recognized HLA-A26-restricted CD8+ T-cell epitope targeting the functionally constrained hepatitis C virus NS5A/5B cleavage site. *J Gen Virol* 2007; **88**: 1986-1991
 - 97 **Gaudieri S**, Rauch A, Park LP, Freitas E, Herrmann S, Jeffrey G, Cheng W, Pfafferoth K, Naidoo K, Chapman R, Battagay M, Weber R, Telenti A, Furrer H, James I, Lucas M, Mallal SA. Evidence of viral adaptation to HLA class I-restricted immune pressure in chronic hepatitis C virus infection. *J Virol* 2006; **80**: 11094-11104
 - 98 **Timm J**, Li B, Daniels MG, Bhattacharya T, Reyor LL, Allgaier R, Kuntzen T, Fischer W, Nolan BE, Duncan J, Schulze Zur Wiesch J, Kim AY, Frahm N, Brander C, Chung RT, Lauer GM, Korber BT, Allen TM. Human leukocyte antigen-associated sequence polymorphisms in hepatitis C virus reveal reproducible immune responses and constraints on viral evolution. *Hepatology* 2007; **46**: 339-349
 - 99 **Neumann-Haefelin C**, McKiernan S, Ward S, Viazov S, Spangenberg HC, Killinger T, Baumert TF, Nazarova N, Sheridan I, Pybus O, von Weizsäcker F, Roggendorf M, Kelleher D, Klennerman P, Blum HE, Thimme R. Dominant influence of an HLA-B27 restricted CD8+ T cell response in mediating HCV clearance and evolution. *Hepatology* 2006; **43**: 563-572
 - 100 **Seifert U**, Liermann H, Racanelli V, Halenius A, Wiese M, Wedemeyer H, Ruppert T, Rispeter K, Henklein P, Sijts A, Hengel H, Kloetzel PM, Rehmann B. Hepatitis C virus mutation affects proteasomal epitope processing. *J Clin Invest* 2004; **114**: 250-259
 - 101 **Kimura Y**, Gushima T, Rawale S, Kaumaya P, Walker CM. Escape mutations alter proteasome processing of major histocompatibility complex class I-restricted epitopes in persistent hepatitis C virus infection. *J Virol* 2005; **79**: 4870-4876
 - 102 **Meyer-Olson D**, Shoukry NH, Brady KW, Kim H, Olson DP, Hartman K, Shintani AK, Walker CM, Kalams SA. Limited T cell receptor diversity of HCV-specific T cell responses is associated with CTL escape. *J Exp Med* 2004; **200**: 307-319
 - 103 **Urbani S**, Amadei B, Cariani E, Fisicaro P, Orlandini A, Missale G, Ferrari C. The impairment of CD8 responses limits the selection of escape mutations in acute hepatitis C virus infection. *J Immunol* 2005; **175**: 7519-7529
 - 104 **Grabowska AM**, Lechner F, Klennerman P, Tighe PJ, Ryder S, Ball JK, Thomson BJ, Irving WL, Robins RA. Direct ex vivo comparison of the breadth and specificity of the T cells in the liver and peripheral blood of patients with chronic HCV infection. *Eur J Immunol* 2001; **31**: 2388-2394
 - 105 **He XS**, Rehmann B, López-Labrador FX, Boisvert J, Cheung R, Mumm J, Wedemeyer H, Berenguer M, Wright TL, Davis MM, Greenberg HB. Quantitative analysis of hepatitis C virus-specific CD8(+) T cells in peripheral blood and liver using peptide-MHC tetramers. *Proc Natl Acad Sci USA* 1999; **96**: 5692-5697
 - 106 **Koziel MJ**, Dudley D, Wong JT, Dienstag J, Houghton M, Ralston R, Walker BD. Intrahepatic cytotoxic T lymphocytes specific for hepatitis C virus in persons with chronic hepatitis. *J Immunol* 1992; **149**: 3339-3344
 - 107 **Penna A**, Missale G, Lamona V, Pilli M, Mori C, Zanelli P, Cavalli A, Elia G, Ferrari C. Intrahepatic and circulating HLA class II-restricted, hepatitis C virus-specific T cells: functional characterization in patients with chronic hepatitis C. *Hepatology* 2002; **35**: 1225-1236
 - 108 **Wong DK**, Dudley DD, Afdhal NH, Dienstag J, Rice CM, Wang L, Houghton M, Walker BD, Koziel MJ. Liver-derived CTL in hepatitis C virus infection: breadth and specificity of responses in a cohort of persons with chronic infection. *J Immunol* 1998; **160**: 1479-1488
 - 109 **Neumann-Haefelin C**, Thimme R. Impact of the genetic restriction of virus-specific T-cell responses in hepatitis C virus infection. *Genes Immun* 2007; **8**: 181-192
 - 110 **Bhattacharya T**, Daniels M, Heckerman D, Foley B, Frahm N, Kadie C, Carlson J, Yusim K, McMahon B, Gaschen B, Mallal S, Mullins JI, Nickle DC, Herbeck J, Rousseau C, Learn GH, Miura T, Brander C, Walker B, Korber B. Founder effects in the assessment of HIV polymorphisms and HLA allele associations. *Science* 2007; **315**: 1583-1586
 - 111 **McKiernan SM**, Hagan R, Curry M, McDonald GS, Kelly A, Nolan N, Walsh A, Hegarty J, Lawlor E, Kelleher D. Distinct MHC class I and II alleles are associated with hepatitis C viral clearance, originating from a single source. *Hepatology* 2004; **40**: 108-114
 - 112 **Wedemeyer H**, Mizukoshi E, Davis AR, Bennink JR, Rehmann B. Cross-reactivity between hepatitis C virus and Influenza A virus determinant-specific cytotoxic T cells. *J Virol* 2001; **75**: 11392-11400
 - 113 **Kennedy PT**, Urbani S, Moses RA, Amadei B, Fisicaro P, Lloyd J, Maini MK, Dusheiko G, Ferrari C, Bertolotti A. The influence of T cell cross-reactivity on HCV-peptide specific human T cell response. *Hepatology* 2006; **43**: 602-611
 - 114 **Thio CL**, Gao X, Goedert JJ, Vlahov D, Nelson KE, Hilgartner MW, O'Brien SJ, Karacki P, Astemborski J, Carrington M, Thomas DL. HLA-Cw*04 and hepatitis C virus persistence. *J Virol* 2002; **76**: 4792-4797
 - 115 **Chuang WC**, Sarkodie F, Brown CJ, Owusu-Ofori S, Brown J, Li C, Navarrete C, Klennerman P, Allain JP. Protective effect of HLA-B57 on HCV genotype 2 infection in a West African population. *J Med Virol* 2007; **79**: 724-733
 - 116 **Stephens HA**. HIV-1 diversity versus HLA class I polymorphism. *Trends Immunol* 2005; **26**: 41-47
 - 117 **Neumann-Haefelin C**, Blum HE, Chisari FV, Thimme R. T cell response in hepatitis C virus infection. *J Clin Virol* 2005; **32**: 75-85

S- Editor Ma N L- Editor Rippe RA E- Editor Ma WH

TOPIC HIGHLIGHT

Robert Thimme, MD, Professor, Series Editor

Memory CD8+ T cell differentiation in viral infection: A cell for all seasons

Henry Radziewicz, Luke Uebelhoer, Bertram Bengsch, Arash Grakoui

Henry Radziewicz, Luke Uebelhoer, Arash Grakoui, Emory Vaccine Center and Department of Microbiology and Immunology; Department of Medicine, Emory University School of Medicine, Atlanta, GA 30322, United States
Bertram Bengsch, Department of Medicine, University Hospital Freiburg, Freiburg 79106, Germany

Supported by NIH National Center for Research Resources K12 RR017643 and NIH K08 AI072191 (HR), the National Institutes of Health through the Grand Challenges in Global Health Initiative, Cancer Research Institute Investigator Award, Woodruff Health Sciences Fund, Yerkes Research Center Base Grant RR-00165 and NIH AI070101 (AG)

Correspondence to: Arash Grakoui, PhD, Emory University School of Medicine, 954 Gatewood Road, NE, Atlanta, GA 30329, United States. arash.grakoui@emory.edu

Telephone: +1-404-7275850 Fax: +1-404-7277768

Received: June 26, 2007 Revised: July 9, 2007

Abstract

Chronic viral infections such as hepatitis B virus (HBV), hepatitis C virus (HCV) and human immunodeficiency virus (HIV) are major global health problems affecting more than 500 million people worldwide. Virus-specific CD8+ T cells play an important role in the course and outcome of these viral infections and it is hypothesized that altered or impaired differentiation of virus-specific CD8+ T cells contributes to the development of persistence and/or disease progression. A deeper understanding of the mechanisms responsible for functional differentiation of CD8+ T cells is essential for the generation of successful therapies aiming to strengthen the adaptive component of the immune system.

© 2007 WJG. All rights reserved.

Key words: Viral infection; Hepatitis C virus; Memory T cell phenotype; Differentiation

Radziewicz H, Uebelhoer L, Bengsch B, Grakoui A. Memory CD8+ T cell differentiation in viral infection: A cell for all seasons. *World J Gastroenterol* 2007; 13(36): 4848-4857

<http://www.wjgnet.com/1007-9327/13/4848.asp>

INTRODUCTION

The adaptive immune response is characterized by the

ability to respond specifically and quickly to antigens that the host has encountered previously. Virus-specific CD8+ T cells critical in this response can be divided into naïve, effector and memory CD8+ T cells. In the strictest sense, the memory response should be maintained in the absence of antigen, poised to respond quickly, specifically, and with sufficient amplitude to protect the host from repeated infection by a previously encountered pathogen^[1,2]. The ability to survive in the absence of antigen differentiates memory T cells from effector cells that exist at the peak of the immune response, while antigen is present. However, in the context of viral infection, differentiation from effector T cells into memory cells may differ depending on the nature of the pathogen.

Many viral infections are acutely cleared by the immune response, whereas others result in persistent infection and are associated with altered differentiation of host T cells. For example, antigen-specific CD8+ T cells isolated from persons with resolved infections such as influenza (Flu) or respiratory syncytial virus (RSV) clearly represent functional memory. With “latent” infections such as Epstein-Barr virus (EBV) or cytomegalovirus (CMV) where low level of virus may still be intermittently present, a strict definition of memory may not be appropriate for these antigen-specific CD8+ T cells^[2]. In fact, van Leeuwen *et al*^[3] proposed to classify these cells as “resting vigilant effector cells” due to their ability to continuously control the latent virus. Currently, neither the frequency nor mechanism of re-encounter with antigen after resolved, primary EBV or CMV infection is well understood. For chronic viral infections such as HIV, hepatitis B virus (HBV) and hepatitis C virus (HCV), isolated antigen-specific CD8+ T cells also may not represent true memory T cells, but rather effector-type cells, or perhaps a population comprising effector and memory cells.

The criteria used to define the differentiation of virus-specific T cells are complicated. In mouse models of viral infection, time after experimental infection is often used to delineate effector T cells from memory T cells. For example, after experimental LCMV infection and clearance, d 40+ has been set as a time at which stable memory CD8+ T cells can be isolated, with many memory qualities being acquired between d 8 and 21 post infection^[4,5]. In addition to time-after-infection, a number of surface antigen markers have been used to differentiate effector CD8+ T cells from memory CD8+ T cells, and to differentiate subsets of memory cells that possess varied levels of differentiation and function. Activation markers

such as CD38 and HLA-DR are often used to identify effector cells which up-regulate these markers during an immune response and which are generally not expressed on quiescent memory cells. Memory cells that re-encounter antigen re-express these activation markers^[6]. Surface expression of the IL-7 receptor- α (CD127) is also used to differentiate effector from memory cells. Naïve and memory cells require an ability to survive and proliferate in the absence of antigen, a process called “homeostatic proliferation”. Cytokines, such as IL-7, and signaling through CD127 are critical in this process^[7]. Naïve cells exposed to antigen decrease expression of CD127 on their cell surface (effector phenotype). Once antigen is cleared, CD127 is re-expressed on memory cells and enables their maintenance. This re-expression characterizes them as memory cells. However, CD127 expression may not be a fool-proof marker of memory, since some subsets of CD8+ T cells identified as memory CD8+ T cells express only low levels of CD127, particularly in latent/chronic viral infections. Whether these cells represent true memory in the strict sense of the definition, or rather represent a population of effectors amidst memory cells is not yet fully elucidated. Finally, immediate cytolytic activity may be the best way to differentiate effector from memory T cells, though some memory cell types also possess immediate cytolytic activity albeit at lower levels^[8].

Improved understanding of functional memory CD8+ T cell development and the identification of unique phenotypic markers of memory CD8+ T cells could be helpful in vaccine development for viral infections where adaptive immune responses play an important role in control and/or clearance (HIV, HBV, HCV). In theory, if efficient memory T cell responses could be induced by vaccination, protective immunity could be achieved. Additionally, further enlightenment into the transition from effector CD8+ T cell into memory CD8+ T cell could aid in the discovery and use of immune modulating therapies that might heighten the response to vaccination. As an example, adoptive transfer studies in mice of antigen-specific CD8+ CD127+ T cells taken during the effector phase of the immune response to LCMV infection have identified CD127 as a marker of CD8+ T cells able to control virus upon re-infection^[5,9]. Therefore, it can be hypothesized that vaccine induction of CD127 expression on CD8+ T cells may be beneficial and should be a goal of any effective vaccine for chronic viral infections. In this review, we will summarize current knowledge of antiviral CD8+ T cell differentiation with a focus on persistent infections such as HCV.

MODELS OF MEMORY CD8+ T CELL DIFFERENTIATION

Several models have been proposed to describe the differentiation of CD8+ T cells from naïve cells to memory cells^[10,11]. In the “linear” or “progressive” model, naïve cells undergo an effector T cell phase prior to developing into memory cells, and all memory T cells are direct descendents of effector cells. This model posits that memory T cells do not develop until antigen is

markedly reduced or eliminated. In the “divergent model”, a stimulated naïve T cell gives rise to either an effector or memory T cell. In this model, naïve T cells can directly give rise to memory T cells without going first through an effector phase. The “decreasing-potential” model accounts for scenarios where antigen persists after primary infection and posits that naïve T cells differentiate into effector cells first. If antigen is cleared early after infection, functional memory T cells develop. If antigen persists, the function of the effectors is sequentially impaired and memory CD8+ T cell development is compromised. Eventually, persistent antigen leads to a non-functional effector cell and eventual cell death by apoptosis. As in the linear model, functional memory cells do not develop until antigen is cleared.

Subsets within the memory CD8+ T cell compartment have been segregated based on markers other than CD127. Sallusto *et al.*^[12] utilized the expression of the lymph node homing receptor, CCR7, and a transmembrane phosphatase involved in T cell signaling, CD45RA, to distinguish central memory (CCR7+ CD45RA-) and effector memory (CCR7-CD45RA- and CCR7-CD45RA+) CD8+ T cell populations^[12]. Central memory cells were characterized by rapid proliferation after antigenic stimulation, while effector memory cells were more capable of immediate effector functions^[12]. In theory, central memory cells are most capable of surveying lymph nodes and responding to antigen with enhanced proliferative capacity, while effector memory cells are more capable of migrating into tissues and exacting immediate effector functions. Appay *et al.*^[13] have proposed another model of CD8+ T cell differentiation during chronic or persistent infection. Studying antigen-specific CD8+ T cells during several different viral infections, they hypothesized that there is a progressive memory differentiation based on differential expression of CD27 and CD28 co-stimulatory molecules^[13]. They defined early (CD27+, CD28+), intermediate (CD27+, CD28-) and late memory CD8+ T cell subsets of virus-specific cells^[13]. The early subset had the greatest proliferative capacity while the intermediate and late subsets had progressively greater cytotoxic potential^[13]. Furthermore, the late subset also expressed CD57, a marker of replicative senescence^[13].

Recently, Romero *et al.*^[14] combined the phenotypic markers used by Sallusto *et al.*^[12] and Appay *et al.*^[13] to further dissect the memory CD8+ T cell pool. They identified four subsets within the effector memory (CD45RA-, CCR7-) pool based on differential staining of CD27 and CD28. Interestingly, the different subgroups differed not only phenotypically, but showed a progressive reduction in telomere length coinciding with a progressive increase in cytotoxic molecules (granzyme B, perforin). Their model lends support to the idea that there is a progressive up-regulation of cytolytic activity and a stepwise loss of CCR7, CD28 and CD27 during the differentiation process. They showed that CD8+ T cells specific for a resolved infection (Flu) consisted of both a central memory (CCR7+, CD45RA-, CD27+ and CD28+) population and a sub-population of effector memory cells termed EM1 (CCR7-, CD45RA-, CD27+ and CD28+).

They hypothesized that this effector memory population, which has only a low expression of the lymphocyte homing receptor, CCR7, confers memory functions and provides surveillance in peripheral tissues^[14].

ACUTE VIRAL INFECTION

Effector phase

In the classic understanding of an adaptive T cell immune response, there is an initial massive expansion of antigen-specific T cells, followed by a period of marked contraction as the pathogen is cleared. This period of expansion and contraction can be referred to as the “acute” or “effector” phase of the immune response. Exceptions to this paradigm likely exist following chronic or latent infections, for example, in CMV infection of humans, where the contraction phase may be more limited^[3]. During this process of expansion and contraction, functional memory cells are formed and persist to protect the host from future infection. Upon infection of mice with LCMV, rare naïve T cells specific for cognate antigen increase exponentially within secondary lymphoid tissues^[15,16]. The responding T cells in this “clonal burst” clear the infection *via* dissemination to non-lymphoid tissues (common sites of infection), the secretion of anti-microbial cytokines such as interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α), and direct lysis of infected host cells^[17-19]. Interestingly, no antigenic stimulation is needed after the initial clonal stimulation, meaning that daughter-cell expansion and differentiation into a memory population occurs in an antigen-independent manner^[20-22]. However, mediation of effector functions, such as cytokine production and killing, is dependent upon contact with antigen bearing targets. In situations where virus is successfully eliminated, > 90% of effector T-cells undergo apoptosis (contraction), and a small population of CD127+ surviving cells undergo further differentiation from an activated phenotype to a resting phenotype to generate a long-lived memory pool that is ready to respond more rapidly upon secondary infection^[5,10]. This pool is maintained in the absence of antigen, and is characterized by IL-7 and IL-15-dependent homeostatic proliferation resulting in relatively constant numbers of CD8+ T cells^[23-25]. During the progression from naïve to effector to memory cells, the homing potential of these cells changes. Upon differentiation to effectors, CD8+ T cells down-regulate lymphoid homing molecules such as CD62L and CCR7, and begin to migrate to nonlymphoid effector sites. Eventually, these lymphoid homing molecules are gradually up-regulated, giving cells the ability to home to lymphoid tissues. This dichotomy of homing potential has prompted researchers to further define memory cells into effector memory or central memory subsets, with the latter having lymphoid homing potential^[26,27].

Much of what we know about memory T cell differentiation is from murine models of infection since identifying humans in the acute phase of viral infection is often difficult, and time from acquisition of infection is rarely precisely known (exceptions described below for accidental infection). Additionally, for some viral infections the acute phase of infection may be relatively

short in duration, while for other infections the “acute” phase may last for weeks to months (HCV). Nevertheless, studying the phenotype of antigen-specific CD8+ T cells during different human acute viral infections has shown that there is a remarkable similarity in terms of surface marker expression and function. During acute infectious mononucleosis, EBV-specific CD8+ T cells show massive expansion (up to 44% of total CD8+ T cells in peripheral blood), and the majority express the activation markers, HLA-DR and CD38, and the memory marker CD45RO (also CD45RA low), but have down-regulated the lymphocyte homing molecule CD62L^[28]. CD28 expression on EBV-specific cells has been shown to range from 9% to 86% depending on the donor, with CD57 expression ranging from 2% to 37%. Appay *et al*^[13] have also shown that during acute infection, some EBV-specific CD8+ T cells express the proliferation marker Ki67. Additionally, these cells were prone to apoptosis since they expressed minimal levels of the anti-apoptotic factor Bcl-2^[13].

In an individual with acute CMV infection, Carmichael *et al*^[29] showed that at the peak of clinical symptoms (3 wk after symptom onset), 80% of CMV-specific CD8+ T cells were CD45RO high, CD28 negative, and CCR7 negative. Studying primary CMV infection after kidney transplantation in humans, Gamadia *et al*^[30] showed that CMV-specific CD8+ T cells evaluated at wk 31 after first positive CMV PCR, were nearly all CD27 positive, but with mixed expression of CD28 (54%). Nearly all expressed CD45RO (minimal CD45RA), and most were CCR7 negative (91%)^[30]. They expressed significant levels of Ki67 (78%), granzyme B (93%) and perforin (100%)^[30]. Few of these acute CMV-specific CD8+ T cells expressed CD127 (1%)^[30].

Other viruses known to cause more persistent infections including HIV, HBV, and HCV, still show characteristic effector T cell development in the early stages after acute infection. For example, in primary HIV infection, nearly all HIV-specific CD8+ T cells expressed CD38 and approximately 11%-41% were Ki67 positive^[13,31]. There was minimal Bcl-2 expression, which correlated inversely with CD38 expression^[13]. Very early after HIV infection, the majority of HIV-specific CD8+ T cells also expressed CD27 and approximately 40% expressed CD28^[31].

Evaluation of 5 persons with acute HBV infection showed that the majority of HBV-specific CD8+ T cells were HLA-DR positive (92%-98%) and CD45RO positive (95%-100%), and most were CCR7 and CD45RA negative, again consistent with an effector phenotype^[32]. The majority expressed CD27, and similar to HIV, 40%-50% expressed CD28^[32]. The frequency of CD127 expression on these antigen-specific cells was also very low^[33].

The phenotype of HCV-specific CD8+ T cells from the peripheral blood of patients evaluated during the acute phase of HCV infection also showed the characteristic expression patterns of effector T cells^[13,34]. Studying 9 patients with acute HCV infection, Lechner *et al*^[34] demonstrated that during acute infection, the activation marker CD38 was up-regulated on HCV specific CD8+ lymphocytes from all patients irrespective of their clinical outcome. By wk 20 after the acute phase, there was a loss

of expression of this activation marker on HCV-specific CD8+ T cells^[34]. Similarly, HLA class II was elevated early during infection and decreased over time^[34]. Studying five health care workers exposed to HCV via accidental needle stick, Thimme *et al.*^[35] demonstrated that HCV-specific CD8+ T cells detectable from a patient with spontaneous viral clearance expressed CD38 on wk 8 and 10 after infection, but by wk 12 and thereafter they were CD38 negative^[35]. CD38 expression correlated with hepatitis, as measured by ALT level^[35]. Interestingly, these activated cells were unable to produce IFN- γ when stimulated by cognate peptide *in vitro*, and the appearance of HCV-specific, IFN- γ producing CD8+ T cells coincided with the disappearance of CD38 expression^[35]. Lechner *et al.*^[36] studied acute infection in one patient, and noted that in addition to increased expression of CD38 and HLA class II on HCV-specific CD8+ T cells early during infection, CCR5 expression was also maximal during the first 20 wk. During the acute phase, CD127 expression was minimal on HCV-specific CD8+ T cells^[37,38].

In summary, for the majority of these viral infections, during the acute phase, there is an increase in expression on antigen-specific CD8+ T cells of activation and proliferation markers, and a decrease in lymph node homing molecules and CD127 expression. Cytolytic molecules are increased but anti-apoptotic factors are decreased. In general, CD27 expression remains present and there are intermediate levels of CD28 expression.

VIRAL PERSISTENCE OR CLEARANCE

Memory phase

While acute models of infection in mice have provided much phenotypic and functional insight into memory CD8+ T cell generation, chronic models of infection have shed light on the inadequacies of cellular responses, and how the resulting persistence of antigen load can affect the differentiation and function of these antigen-specific cells. Indeed, the course of memory CD8+ T cell differentiation during chronic infections can vary greatly from that which is seen in acute infections, including unique tissue distribution of antigen-specific T cells, dominance of T cell populations that normally have subdominant specificities, and even gradual exhaustion or deletion of entire T cell populations from the repertoire^[25]. The hallmark of differentiation in chronic infection is a stepwise loss of T cell effector functions that becomes more severe as time progresses, as opposed to the gain of effector functions that is seen in acute infections^[39]. This “exhaustion” can be broken into several categories, corresponding to the severity of impairment of effector function and proliferative potential. Initial antigen stimulation leads to CD8+ T cells that are functionally competent in that they can produce IFN- γ , TNF- α , IL-2, are cytolytic, and have robust proliferative capacity. However, if virus persists, these cells become partially exhausted, losing their ability to lyse target cells and produce IL-2 first, followed by decreased TNF- α production^[40,41]. Interestingly, cells that are partially exhausted may still have the ability to proliferate and produce IFN- γ , albeit with reduced efficiency. As antigen persists, cells may become fully

exhausted, completely losing both effector functions and the ability to proliferate^[39,41,42]. It has also been shown in chronic LCMV infection that deletion of antigen-specific CD8+ T cells can occur if antigen load is both extremely high and persistent^[41,43]. Additionally, CD4+ T cells play an important role in the chronic exhaustion of CD8+ T cell responses, both throughout infection and in the priming of cells during the acute phase of infection. Unlike antigen load, CD4+ T cell help is directly related to the functionality of the CD8+ T cell effector response: the absence of this help leads to a more rapid and severe progression to the exhausted phenotype^[25].

Unlike the similarities in the range of phenotype of antigen-specific cells seen during the acute phase of different viral infections in humans, the phenotype of antigen-specific cells isolated during different latent/chronic viral infections is more diverse. In the chronic phase of HCV infection, Lechner *et al.*^[36] were unable to detect the activation markers CD38 or HLA class II expression on any HCV-specific CD8+ T cells. This is different from HIV infection, where during chronic infection a proportion of HIV-specific CD8+ T cells in the blood expressed CD38 and HLA-DR^[44]. Appay *et al.*^[13] compared HIV, CMV, EBV and HCV-antigen specific CD8+ T cells taken from blood during the latent/chronic stage of infection. Though the majority of antigen-specific CD8+ T cells for these viral infections had all lost evidence for activation (minimal CD38 expression) and proliferation (minimal Ki67 expression), and had up-regulated the survival factor, Bcl-2, the expression of CD27 and CD28 differed^[13]. The majority of HCV-specific CD8+ T cells expressed both CD27 (90%) and CD28 (90%), while EBV-specific CD8+ T cells had comparable levels of CD27 expression but lower CD28 expression (60%). HIV-specific CD8+ T cells had relatively high levels of CD27 expression (80%), but very low levels of CD28 expression (10%). Finally, the majority of CMV-specific CD8+ T cells had relatively low expression of both CD27 (30%) and CD28 (20%). This comparison of differentiation in the chronic/latent phase of multiple infections prompted the authors to label EBV- and HCV-specific CD8+ T cells “early”, HIV-specific CD8+ T cells “intermediate” and CMV-specific CD8+ T cells as “late” differentiated. CCR7 expression was low on HIV-, CMV- and EBV-specific CD8+ T cells^[13], whereas others have shown that peripheral HCV-specific CD8+ T cells are largely CCR7+^[45,46]. Others have shown that EBV-specific CD8+ T cells may be better represented as a mixture of effector memory and central memory cells (or early and late differentiation states) as delineated by differential staining of CCR7 and CD45RA^[47].

For cleared viral infections such as influenza and RSV, antigen-specific CD8+ T cells resembled these HCV and EBV-specific cells in that they were mostly CD27+ and CD28+^[48,49]. RSV-specific CD8+ T cells were mostly CCR7 negative (92%)^[49] in contrast with influenza, where a greater frequency of specific CD8+ T cells were CCR7 positive^[49]. The range of CCR7 expression on both RSV and Flu, however, were broad among different patients in this study (0%-71% for RSV and 0%-57% for Flu)^[49].

Similar to the mouse, CD127 expression appears to be

Table 1 Phenotype of antigen-specific CD8⁺ T cells found in blood and tissue during different viral infections

Virus	Peripheral blood phenotype	Tissue	Tissue phenotype
Flu	CD27 ⁺ CD28 ⁺ CCR7 ⁺ CD127 ⁺	Lung	CD27 ⁻ CD28 ⁻
RSV	CD27 ⁺ CD28 ⁺ CCR7 ⁻ CD127 ⁺	Lung	CD27 ⁻ CD28 ⁻
EBV	CD27 ⁺ CD28 ⁺ CCR7 ⁻ CD127 ⁻	Tonsil	↑ CD38 ↑ CCR7 ↑ CD127
CMV	CD27 ⁻ CD28 ⁻ CCR7 ⁻ CD127 ⁻ CD45RA ⁺	Tonsil	↑ CD127
HIV	CD27 ⁺ CD28 ⁻ CCR7 ⁻ CD127 ⁻	Rectum	CCR7 ⁻ CD127 ⁻ ↓ perforin
HCV	CD27 ⁺ CD28 ⁺ CCR7 ⁺ CD127 ⁺	Liver	↑ CD69 CCR7 ⁻ CD127 ⁻
HBV	CCR7 ⁺ CD127 ⁺	Liver	↑ HLA-DR

Arrows indicate increase or decrease relative to expression in blood.

associated with memory phenotype and viral clearance of some infections in humans. CD8⁺ T cells isolated from persons with resolved viral infections such as influenza or RSV expressed high levels of CD127^[50]. Similarly, longitudinal analysis of 6 patients with acute resolving HBV infection showed that after viral clearance, CD127 expression increased markedly and correlated with the loss of CD38 and PD-1 expression, acquisition of CCR7 expression and enhanced proliferative capacity^[33]. In contrast, for latent infections (EBV, CMV) and persistent viral infections (HIV) low levels of CD127 have been noted on virus-specific CD8⁺ T cells^[50-52]. However, in EBV infection CD127 expression was higher on cells specific for latent epitopes compared with lytic epitopes^[47]. When we evaluated CD127 expression on peripheral HCV-specific CD8⁺ T cells from patients with chronic HCV infection, we were surprised to find that in the majority of patients, nearly all expressed high CD127 expression despite the high level of antigen present^[53]. This phenotype is reminiscent of resolved infection such as influenza. However, Bengsch *et al*^[37] identified two subsets of patients with chronic HCV: One with HCV-specific CD8⁺ T cells predominantly expressing low levels of CD127 and the other expressing higher levels of CD127. Interestingly, the CD127 low group also had higher level of CD38⁺ frequencies and lower level of CCR7 expression hinting that in this group, re-activation of these cells may have induced the down-regulation of CD127.

Table 1 summarizes the phenotype of antigen-specific cells from the peripheral blood of persons with resolved, latent and chronic viral infections. There is substantial heterogeneity between the different viral infections, as noted in the table.

MECHANISMS OF VARIED MEMORY DIFFERENTIATION IN PERSISTENT OR LATENT INFECTION

Currently, the explanation for the variation in differentiation phenotype seen in the setting of different viral infections is not completely understood, but a number

of hypotheses exist. Clonal expansion, effector functions and memory formation require three signals during the immune response: antigen (signal 1), co-stimulation (signal 2) and cytokine (signal 3)^[54]. Alterations in these signals could contribute to alterations in CD8⁺ T cell phenotype and function. Optimal expansion and function of naïve CD8⁺ T cells required antigen and co-stimulation to be present for approximately 36 h and cytokine (IL-12) present from about h 12 to h 60^[54,55]. These signals launch a complex program of proliferation and differentiation. Given this “autopilot” response^[56], the quality and context of the original signal may have a critical impact on subsequent T cell differentiation^[57]. Differing numbers of naïve precursor cells, antigen loads, cytokine milieu and primary location of infection (lymph node, gut, lung, liver) seen with the different viral infections would offer additional possible explanations for the diversity of these CD8⁺ T cells. In line with this hypothesis, Marzo *et al*^[58] have shown that initial precursor frequency is critical in determining effector and central memory CD8⁺ T cell differentiation. Increasing the input number of antigen-specific CD8⁺ T cells during the primary immune response resulted in increasingly larger populations of central memory cells. Furthermore, effector memory CD8⁺ T cells generated from high or low numbers were fundamentally different, in that cells generated from low initial naïve T cell precursor frequency were unable to interconvert and re-express CD62L^[58].

Even after establishment of persistent viral infection, initial events in naïve T cell proliferation and differentiation likely continue to play a critical role in the varied CD8⁺ T cell differentiation phenotypes that are seen in different viral infections. Vezys *et al*^[59] have recently shown that during persistent viral infection, there is a continuous recruitment of naïve T cells that contributes to the heterogeneity of antiviral CD8⁺ T cells. In their model, antigen-specific memory T cells were not maintained in the presence of antigen without replenishment from thymic emigrants. By induction of a partial hematopoietic chimerism in persistently infected mice using busulfan and congenic bone marrow, they showed that there were variations in the expression of CD27, CD62L, CD127 and bcl-2 between cell populations primed at different times^[59], and that heterogeneity in the memory population was related to this. Their study certainly complicates the current models of T cell differentiation described above, and highlights the dynamic nature of chronic viral infections, even despite relatively stable levels of viral load measured in the peripheral blood of patients with chronic HBV, HCV or HIV.

After initial viral infection and programming of naïve CD8⁺ T cells, downstream events may also impact on CD8⁺ T cell differentiation. Wherry *et al*^[60] have recently shown that in the setting of chronic infection, it is viral antigen and extensive division of virus-specific CD8⁺ T cells that maintains cell numbers, in marked contrast to the slow turnover seen during homeostatic proliferation of memory T cells from cleared viral infection. These cells, in the context of persistent infection, would be expected to display a different phenotype from those isolated from cleared infection, given differences in cell turnover. After

naïve CD8+ T cells are activated and become effector cells, they shortly lose the ability to produce IL-2 upon re-encounter with antigen and co-stimulation, a condition referred to as activation induced non-responsiveness (AINR)^[54]. IL-2 (provided by CD4+ T cells) is able to reverse this state. Co-stimulation via molecules other than CD28, such as OX40 (CD134), and 4-1BB (CD137) may also be critical in providing a stimulation for continued expansion once AINR develops^[54]. Interestingly, we have shown in chronic HCV infection that the co-inhibitory molecule, PD-1, is highly expressed on HCV-specific CD8+ T cells and on total CD8+ T cells at the site of infection in the liver^[53]. It is possible, that a lack of adequate co-stimulation and/or vigorous co-inhibitory signals prevent reversion of AINR and explains the exhaustion and CD8+ T cell deletion seen during this chronic viral infection. In addition to loss of IL-2 production, chronic antigen stimulation eventually also leads to characteristic progressive loss of TNF- α and finally IFN- γ production by cytotoxic CD8+ T cells^[39,57,61]. Since cytokines alone have been shown to induce T cell differentiation^[62] a lack of available cytokine during persistent infection may also explain altered differentiation patterns seen with different viral infections. In a transgenic mouse model, HBV-specific CD8+ T cells are rapidly induced to produce IFN- γ when they enter the liver, but are then rapidly suppressed despite continued antigen^[63]. Suppression of cytokine production was mediated by the co-inhibitor, PD-1, since blockade of PD-1 led to a delay in the suppression of IFN- γ producing cells^[63].

The potency of the pathogen and the antigen load may also influence memory differentiation. In a mouse model of infection, reducing the stimulation of CD8+ T cells by using an attenuated pathogen led to primarily a central memory subset, while infection with a more virulent pathogen led to effector cell development^[64]. Tussey *et al*^[65] compared the phenotype of HIV-specific CD8+ T cells in the setting of viral control (using anti-retroviral medication) and in the setting of uncontrolled viremia. They showed that the phenotype of these antigen-specific cells differed based on the level of viremia, and hypothesized that the level of antigen burden determined the differentiation state. Similarly, Papagno *et al*^[66] showed that in HIV infection excessive levels of antigen stimulation as determined by level of HIV disease progression lead to a progressive differentiation toward a state of replicative senescence. Given these studies, the importance of the quantity and duration of persistent antigen would seem to be very important factors leading to varied differentiation programs. However, in chronic HCV infection, antigen burden is large, with viral loads often on the order of 10^6 - 10^7 , yet as previously described, differentiation of HCV-specific CD8+ T cells is more limited and these cells have been termed “early”. Viral escape is one possibility for the lack of progressive differentiation, yet when we sequenced HCV isolated from the peripheral blood at the epitope specific for the tetramer used to identify these “early” differentiated HCV-specific CD8+ T cells, no mutations were seen^[53]. Another possibility to explain variation in differentiation phenotype is differences in innate signals among different infections,

and differences in these signals occurring at early and late time-points during persistent infection^[67]. This could be particularly relevant for HCV infection, since it has been shown that the NS3-4A serine protease of HCV degrades the adapter molecule, Cardif^[68], and thereby interferes with the RIG-I mediated process of innate recognition of dsRNA. The “early” differentiated phenotype of HCV-specific CD8+ T cells during chronic infection could be a reflection of this impairment in innate signaling. Pulendran *et al*^[67] has also speculated that during the early stages of an immune response, highly stimulatory DC subsets might deliver strong TCR signals favoring effector T cell differentiation, while at later stages, a milder form of T cell stimulation by less stimulatory DC subsets could favor the development of central memory T cells.

Location, location, location

Finally, a perhaps somewhat overlooked cause for differences in memory phenotype in different viral infections may be related to the anatomic location of the different viral infections, and to discrepancies between the active site of infection (liver, lung, *etc.*) and the site from which cells were obtained for study. Mice infected with vesicular stomatitis virus (VSV) developed memory cells with functional differences in cytolytic activity based on their location in either tissue (lung, liver, small intestine) or secondary lymphoid organs, highlighting the importance of anatomic location in type of isolated memory cell subset^[69]. Masopust *et al*^[70] have also shown that virus specific intraepithelial lymphocytes in the gut resemble neither central nor effector memory CD8+ T cells isolated from spleen or blood by almost all properties examined, including effector function, differentiation, homing receptors and cell cycle. In fact, memory CD8+ T cells changed phenotype upon change of location^[70].

As noted above, we were surprised to find a phenotype of HCV-specific CD8+ T cells that resembled the phenotype of a resolved infection (Flu) in terms of high expression of CD127, CCR7, CD28 and CD27. However, analysis of antigen-specific cells at the site of active infection, the liver, revealed that nearly all of the antigen-specific CD8+ T cells displayed a different phenotype, with low CD127 expression^[37,53]. Similarly, Accapezzato *et al*^[71] have shown that HCV-specific CD8+ T cells isolated from liver express markedly lower CCR7 expression in comparison with peripheral blood. HCV-specific CD8+ T cells in the liver also displayed an activated phenotype with elevation of expression of the early activation marker CD69^[72]. We hypothesize that a lack of exposure to antigen by the peripheral CD8+ T cells enabled up-regulation of CD127 and CD62L and memory formation, since exposure to cognate peptide *in vitro* induced a down-regulation of CD127 on these same cells. The effector-like cells isolated from the liver were likely actively involved in the immune response occurring at the site of infection. Similar to our findings with chronic HCV infection, peripheral blood HBV-specific CD8+ T cells expressed high levels of CD127 during chronic HBV infection despite high levels of antigen load^[73]. Analysis of HBV-specific CD8+ T cells in the liver of patients with resolving HBV infection showed that a greater frequency

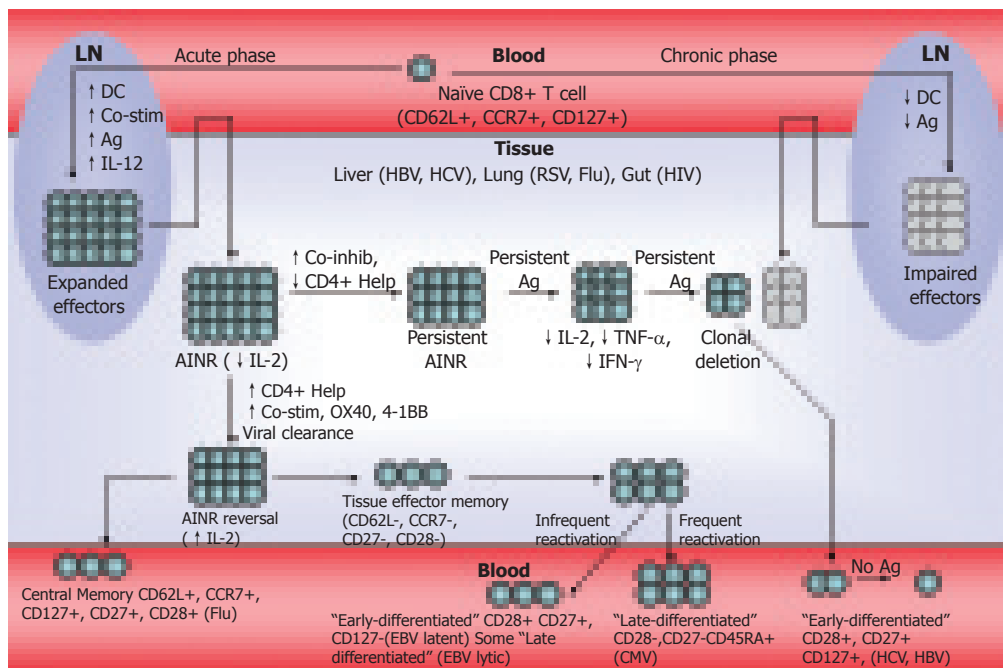


Figure 1 Tissue-specific model of antigen-specific memory CD8⁺ T cell differentiation.

were activated, as demonstrated by HLA-DR expression, in comparison with the peripheral blood^[74]. It will be interesting to further analyze the phenotype of these HBV-specific cells at the site of infection.

In addition to the liver, human memory CD8⁺ T cells at other locations are clearly influenced by the anatomic site where they reside. De Bree *et al*^[75] have compared Flu and RSV-specific CD8⁺ T cells in the lungs and peripheral blood. They found that the lung contained markedly higher frequencies of Flu- and RSV-specific cells compared with the peripheral blood^[75]. A substantial percentage of these lung residing antigen-specific CD8⁺ T cells had progressed to a relatively late differentiation phenotype with low expression of CD28 and CD27^[75]. EBV-specific CD8⁺ T cells isolated from the tonsils of long-term carriers were more likely to express the activation marker CD38 and CD103, an integrin induced by epithelium-derived cytokine TGF- β ^[76]. Both EBV (lytic)- and CMV-specific CD8⁺ T cells in the tonsils were shown to have increased CD127 expression in comparison with peripheral blood^[47]. In HIV infection, rectal HIV-specific CD8⁺ T cells expressed a similar effector memory phenotype as in the peripheral blood (CCR7⁻), but expressed minimal perforin, unlike in the peripheral blood where as many as 23% of Gag-specific CD8⁺ T cells expressed perforin^[77]. Table 1 summarizes the phenotype differences of viral-specific CD8⁺ T cells between peripheral blood and tissue (liver, lung, tonsil, gut). Clearly, there is much to be learned about memory T cell differentiation by further evaluation of T cells residing in tissues other than peripheral blood.

A MODEL OF TISSUE DEPENDENT MEMORY DIFFERENTIATION

So, how might the variation in differentiation phenotypes of antigen-specific CD8⁺ T cells from different viral infections and from different anatomic locations look? A

schematic is shown in Figure 1. During the acute phase of infection, naïve CD8⁺ T cells surveying the lymph node (LN) may encounter antigen presented by activated dendritic cells (\uparrow DC) in the context of high antigen concentration (\uparrow Ag), significant co-stimulatory signal (\uparrow Co-stim), and cytokine (IL-12 or type I interferon) (\uparrow IL-12). This leads to an expansion of competent effectors that have decreased lymph node homing receptors (CD62L, CCR7) and migrate to sites of infection (e.g. liver, lung, gut). These cells lose the ability to produce IL-2 (activation induced non-responsiveness, AINR)^[54]. With proper CD4⁺ T cell help (\uparrow CD4⁺ Help) or other co-stimulatory signals, such as via 4-1BB (\uparrow Co-stim, OX40, 4-1BB), these cells maintain function and succeed in clearing virus. One population of cells forms central memory cells (CD62L⁺, CCR7⁺, CD127⁺, CD45RA⁻) that have up-regulated lymph node homing molecules and are easily detected in the peripheral blood. A second population of effector memory cells is maintained in the tissue (perhaps by homeostatic mechanisms) (CD62L⁻, CCR7⁻, CD27⁻, CD28⁻). For the latent viruses, EBV and CMV, we hypothesize that differences in the frequency and/or location of re-activation offers an explanation for the “early” *vs* “late” phenotype. Perhaps CMV reactivation or even low-level persistence occurs at peripheral sites, and sampling of antigen-specific CD8⁺ T cells from the peripheral blood reflects this (late differentiation). EBV reactivation may be less frequent (and not persistent) and, therefore, a phenotype similar to a resolved infection such as Flu or RSV is sometimes seen (though CD127 expression is diminished). EBV reactivation may also lead to a population of more differentiated cells specific for the lytic epitope. If AINR cannot be reversed, as might be the case with a lack of CD4⁺ T cell help (\downarrow CD4⁺ Help) or via enhanced co-inhibitory signals (\downarrow Co-inhib), there is a progressive loss of function of virus-specific CD8⁺ T cells (\downarrow IL-2, \downarrow TNF- α , \downarrow IFN- γ) eventually leading to clonal deletion. For HCV and HBV infections,

perhaps at the site of infection in the liver, where antigen is present and concentrated, persistent antigen maintains a population of effector-like cells. As Wherry *et al.*^[78] have described in chronic LCMV infection, this cell population would not be maintained without antigen present. These effector cells would be expected to express low levels of CD127 and lymph node homing molecules. Additionally, naïve cells encounter antigen during the chronic phase; however in this setting, DCs are less stimulatory (\downarrow DC) and antigen is lower (\downarrow Ag) than what is seen during acute infection. The resulting impaired effectors contribute to the pool of antigen-specific cells. In the periphery, HCV- and HBV-specific CD8⁺ T cells are not maintained by persistent antigen, but rather proceed to form functional memory T cells and are maintained via homeostatic signals. We and others have found that peripheral blood HCV-specific CD8⁺ T cells expressing CD127 have a capacity for proliferation upon ex vivo antigen encounter similar to other functional memory T cells. For HIV infection (and persistent LCMV infection), where antigen is located in the periphery, these cells are maintained only by the presence of antigen and thereby, display the phenotype of an effector-like cell in the periphery.

CONCLUSION

The phenotype of antigen-specific CD8⁺ T cells persisting during different viral infections is quite varied. This variation may be related to a number of factors including level of antigen persistence, strength of antigen presenting cell interactions, balance of co-stimulatory/co-inhibitory signals, and the influence of the anatomic location of infection. The requirement of continued antigen in the maintenance of virus-specific CD8⁺ T cells during chronic viral infection and continued recruitment of naïve CD8⁺ T cells into the population of antigen-specific cells highlight the dynamic nature of these infections and the cells responding to them. Improved understanding of the relative contribution of each of these factors in the formation of functional memory cells may aid in the development of virus-specific treatments to enhance the immune response to infection or vaccines.

ACKNOWLEDGMENTS

Holly L Hanson, Dave Masopust and Raghavan Chinnadurai for critical reading of the manuscript.

REFERENCES

- 1 Veiga-Fernandes H, Walter U, Bourgeois C, McLean A, Rocha B. Response of naïve and memory CD8⁺ T cells to antigen stimulation *in vivo*. *Nat Immunol* 2000; **1**: 47-53
- 2 Rocha B, Tanchot C. The Tower of Babel of CD8⁺ T-cell memory: known facts, deserted roads, muddy waters, and possible dead ends. *Immunol Rev* 2006; **211**: 182-196
- 3 van Leeuwen EM, de Bree GJ, ten Berge IJ, van Lier RA. Human virus-specific CD8⁺ T cells: diversity specialists. *Immunol Rev* 2006; **211**: 225-235
- 4 Kaech SM, Hemby S, Kersh E, Ahmed R. Molecular and functional profiling of memory CD8 T cell differentiation. *Cell* 2002; **111**: 837-851
- 5 Kaech SM, Tan JT, Wherry EJ, Konieczny BT, Surh CD, Ahmed R. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nat Immunol* 2003; **4**: 1191-1198
- 6 Oxenius A, Günthard HF, Hirschel B, Fidler S, Weber JN, Easterbrook PJ, Bell JL, Phillips RE, Price DA. Direct ex vivo analysis reveals distinct phenotypic patterns of HIV-specific CD8(+) T lymphocyte activation in response to therapeutic manipulation of virus load. *Eur J Immunol* 2001; **31**: 1115-1121
- 7 Fry TJ, Mackall CL. The many faces of IL-7: from lymphopoiesis to peripheral T cell maintenance. *J Immunol* 2005; **174**: 6571-6576
- 8 Wolint P, Betts MR, Koup RA, Oxenius A. Immediate cytotoxicity but not degranulation distinguishes effector and memory subsets of CD8⁺ T cells. *J Exp Med* 2004; **199**: 925-936
- 9 Huster KM, Busch V, Schiemann M, Linkemann K, Kersiek KM, Wagner H, Busch DH. Selective expression of IL-7 receptor on memory T cells identifies early CD40L-dependent generation of distinct CD8⁺ memory T cell subsets. *Proc Natl Acad Sci USA* 2004; **101**: 5610-5615
- 10 Kaech SM, Wherry EJ, Ahmed R. Effector and memory T-cell differentiation: implications for vaccine development. *Nat Rev Immunol* 2002; **2**: 251-262
- 11 Intlekofer AM, Wherry EJ, Reiner SL. Not-so-great expectations: re-assessing the essence of T-cell memory. *Immunol Rev* 2006; **211**: 203-213
- 12 Sallusto F, Lenig D, Förster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 1999; **401**: 708-712
- 13 Appay V, Dunbar PR, Callan M, Klenerman P, Gillespie GM, Papagno L, Ogg GS, King A, Lechner F, Spina CA, Little S, Havlir DV, Richman DD, Gruener N, Pape G, Waters A, Easterbrook P, Salio M, Cerundolo V, McMichael AJ, Rowland-Jones SL. Memory CD8⁺ T cells vary in differentiation phenotype in different persistent virus infections. *Nat Med* 2002; **8**: 379-385
- 14 Romero P, Zippelius A, Kurth I, Pittet MJ, Touvrey C, Iancu EM, Corthesy P, Devedre E, Speiser DE, Rufer N. Four functionally distinct populations of human effector-memory CD8⁺ T lymphocytes. *J Immunol* 2007; **178**: 4112-4119
- 15 Murali-Krishna K, Altman JD, Suresh M, Sourdive DJ, Zajac AJ, Miller JD, Slansky J, Ahmed R. Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. *Immunity* 1998; **8**: 177-187
- 16 Hou S, Hyland L, Ryan KW, Portner A, Doherty PC. Virus-specific CD8⁺ T-cell memory determined by clonal burst size. *Nature* 1994; **369**: 652-654
- 17 Oehen S, Brduscha-Riem K. Differentiation of naïve CTL to effector and memory CTL: correlation of effector function with phenotype and cell division. *J Immunol* 1998; **161**: 5338-5346
- 18 Blattman JN, Antia R, Sourdive DJ, Wang X, Kaech SM, Murali-Krishna K, Altman JD, Ahmed R. Estimating the precursor frequency of naïve antigen-specific CD8 T cells. *J Exp Med* 2002; **195**: 657-664
- 19 Bachmann MF, Barner M, Viola A, Kopf M. Distinct kinetics of cytokine production and cytolysis in effector and memory T cells after viral infection. *Eur J Immunol* 1999; **29**: 291-299
- 20 Mercado R, Vijn S, Allen SE, Kersiek K, Pilip IM, Pamer EG. Early programming of T cell populations responding to bacterial infection. *J Immunol* 2000; **165**: 6833-6839
- 21 van Stipdonk MJ, Lemmens EE, Schoenberger SP. Naïve CTLs require a single brief period of antigenic stimulation for clonal expansion and differentiation. *Nat Immunol* 2001; **2**: 423-429
- 22 Wong P, Pamer EG. Cutting edge: antigen-independent CD8 T cell proliferation. *J Immunol* 2001; **166**: 5864-5868
- 23 Lau JF, Horvath CM. Mechanisms of Type I interferon cell signaling and STAT-mediated transcriptional responses. *Mt Sinai J Med* 2002; **69**: 156-168
- 24 Murali-Krishna K, Lau LL, Sambhara S, Lemonnier F, Altman J, Ahmed R. Persistence of memory CD8 T cells in MHC class I-deficient mice. *Science* 1999; **286**: 1377-1381
- 25 Wherry EJ, Ahmed R. Memory CD8 T-cell differentiation during viral infection. *J Virol* 2004; **78**: 5535-5545
- 26 Weninger W, Crowley MA, Manjunath N, von Andrian UH. Migratory properties of naïve, effector, and memory CD8(+) T

- cells. *J Exp Med* 2001; **194**: 953-966
- 27 **Wherry EJ**, Teichgräber V, Becker TC, Masopust D, Kaech SM, Antia R, von Andrian UH, Ahmed R. Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat Immunol* 2003; **4**: 225-234
 - 28 **Callan MF**, Tan L, Annels N, Ogg GS, Wilson JD, O'Callaghan CA, Steven N, McMichael AJ, Rickinson AB. Direct visualization of antigen-specific CD8+ T cells during the primary immune response to Epstein-Barr virus *In vivo*. *J Exp Med* 1998; **187**: 1395-1402
 - 29 **Wills MR**, Okecha G, Weekes MP, Gandhi MK, Sissons PJ, Carmichael AJ. Identification of naive or antigen-experienced human CD8(+) T cells by expression of costimulation and chemokine receptors: analysis of the human cytomegalovirus-specific CD8(+) T cell response. *J Immunol* 2002; **168**: 5455-5464
 - 30 **Gamadia LE**, Remmerswaal EB, Weel JF, Bemelman F, van Lier RA, Ten Berge IJ. Primary immune responses to human CMV: a critical role for IFN-gamma-producing CD4+ T cells in protection against CMV disease. *Blood* 2003; **101**: 2686-2692
 - 31 **Appay V**, Papagno L, Spina CA, Hansasuta P, King A, Jones L, Ogg GS, Little S, McMichael AJ, Richman DD, Rowland-Jones SL. Dynamics of T cell responses in HIV infection. *J Immunol* 2002; **168**: 3660-3666
 - 32 **Urbani S**, Boni C, Missale G, Elia G, Cavallo C, Massari M, Raimondo G, Ferrari C. Virus-specific CD8+ lymphocytes share the same effector-memory phenotype but exhibit functional differences in acute hepatitis B and C. *J Virol* 2002; **76**: 12423-12434
 - 33 **Boettler T**, Panther E, Bengsch B, Nazarova N, Spangenberg HC, Blum HE, Thimme R. Expression of the interleukin-7 receptor alpha chain (CD127) on virus-specific CD8+ T cells identifies functionally and phenotypically defined memory T cells during acute resolving hepatitis B virus infection. *J Virol* 2006; **80**: 3532-3540
 - 34 **Lechner F**, Gruener NH, Urbani S, Uggeri J, Santantonio T, Kammer AR, Cerny A, Phillips R, Ferrari C, Pape GR, Klennerman P. CD8+ T lymphocyte responses are induced during acute hepatitis C virus infection but are not sustained. *Eur J Immunol* 2000; **30**: 2479-2487
 - 35 **Thimme R**, Oldach D, Chang KM, Steiger C, Ray SC, Chisari FV. Determinants of viral clearance and persistence during acute hepatitis C virus infection. *J Exp Med* 2001; **194**: 1395-1406
 - 36 **Lechner F**, Wong DK, Dunbar PR, Chapman R, Chung RT, Dohrenwend P, Robbins G, Phillips R, Klennerman P, Walker BD. Analysis of successful immune responses in persons infected with hepatitis C virus. *J Exp Med* 2000; **191**: 1499-1512
 - 37 **Bengsch B**, Spangenberg HC, Kersting N, Neumann-Haefelin C, Panther E, von Weizsäcker F, Blum HE, Pircher H, Thimme R. Analysis of CD127 and KLRG1 expression on hepatitis C virus-specific CD8+ T cells reveals the existence of different memory T-cell subsets in the peripheral blood and liver. *J Virol* 2007; **81**: 945-953
 - 38 **Urbani S**, Amadei B, Tola D, Massari M, Schivazappa S, Missale G, Ferrari C. PD-1 expression in acute hepatitis C virus (HCV) infection is associated with HCV-specific CD8 exhaustion. *J Virol* 2006; **80**: 11398-11403
 - 39 **Wherry EJ**, Blattman JN, Murali-Krishna K, van der Most R, Ahmed R. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *J Virol* 2003; **77**: 4911-4927
 - 40 **Appay V**, Nixon DF, Donahoe SM, Gillespie GM, Dong T, King A, Ogg GS, Spiegel HM, Conlon C, Spina CA, Havlir DV, Richman DD, Waters A, Easterbrook P, McMichael AJ, Rowland-Jones SL. HIV-specific CD8(+) T cells produce antiviral cytokines but are impaired in cytolytic function. *J Exp Med* 2000; **192**: 63-75
 - 41 **Fuller MJ**, Zajac AJ. Ablation of CD8 and CD4 T cell responses by high viral loads. *J Immunol* 2003; **170**: 477-486
 - 42 **Zajac AJ**, Blattman JN, Murali-Krishna K, Sourdive DJ, Suresh M, Altman JD, Ahmed R. Viral immune evasion due to persistence of activated T cells without effector function. *J Exp Med* 1998; **188**: 2205-2213
 - 43 **van der Most RG**, Murali-Krishna K, Lanier JG, Wherry EJ, Puglielli MT, Blattman JN, Sette A, Ahmed R. Changing immunodominance patterns in antiviral CD8 T-cell responses after loss of epitope presentation or chronic antigenic stimulation. *Virology* 2003; **315**: 93-102
 - 44 **Ho HN**, Hultin LE, Mitsuyasu RT, Matud JL, Hausner MA, Bockstoe D, Chou CC, O'Rourke S, Taylor JM, Giorgi JV. Circulating HIV-specific CD8+ cytotoxic T cells express CD38 and HLA-DR antigens. *J Immunol* 1993; **150**: 3070-3079
 - 45 **Lauer GM**, Barnes E, Lucas M, Timm J, Ouchi K, Kim AY, Day CL, Robbins GK, Casson DR, Reiser M, Dusheiko G, Allen TM, Chung RT, Walker BD, Klennerman P. High resolution analysis of cellular immune responses in resolved and persistent hepatitis C virus infection. *Gastroenterology* 2004; **127**: 924-936
 - 46 **Lucas M**, Vargas-Cuero AL, Lauer GM, Barnes E, Willberg CB, Semmo N, Walker BD, Phillips R, Klennerman P. Pervasive influence of hepatitis C virus on the phenotype of antiviral CD8+ T cells. *J Immunol* 2004; **172**: 1744-1753
 - 47 **Sauce D**, Larsen M, Leese AM, Millar D, Khan N, Hislop AD, Rickinson AB. IL-7R alpha versus CCR7 and CD45 as markers of virus-specific CD8+ T cell differentiation: contrasting pictures in blood and tonsillar lymphoid tissue. *J Infect Dis* 2007; **195**: 268-278
 - 48 **He XS**, Mahmood K, Maecker HT, Holmes TH, Kemble GW, Arvin AM, Greenberg HB. Analysis of the frequencies and of the memory T cell phenotypes of human CD8+ T cells specific for influenza A viruses. *J Infect Dis* 2003; **187**: 1075-1084
 - 49 **de Bree GJ**, Heidema J, van Leeuwen EM, van Bleek GM, Jonkers RE, Jansen HM, van Lier RA, Out TA. Respiratory syncytial virus-specific CD8+ memory T cell responses in elderly persons. *J Infect Dis* 2005; **191**: 1710-1718
 - 50 **van Leeuwen EM**, de Bree GJ, Remmerswaal EB, Yong SL, Tesselaar K, ten Berge IJ, van Lier RA. IL-7 receptor alpha chain expression distinguishes functional subsets of virus-specific human CD8+ T cells. *Blood* 2005; **106**: 2091-2098
 - 51 **Boutboul F**, Puthier D, Appay V, Pellé O, Ait-Mohand H, Combadière B, Carcelain G, Katlama C, Rowland-Jones SL, Debré P, Nguyen C, Autran B. Modulation of interleukin-7 receptor expression characterizes differentiation of CD8 T cells specific for HIV, EBV and CMV. *AIDS* 2005; **19**: 1981-1986
 - 52 **Paiardini M**, Cervasi B, Albrecht H, Muthukumar A, Dunham R, Gordon S, Radziejewicz H, Piedimonte G, Magnani M, Montroni M, Kaech SM, Weintrob A, Altman JD, Sodora DL, Feinberg MB, Silvestri G. Loss of CD127 expression defines an expansion of effector CD8+ T cells in HIV-infected individuals. *J Immunol* 2005; **174**: 2900-2909
 - 53 **Radziejewicz H**, Ibegbu CC, Fernandez ML, Workowski KA, Obideen K, Wehbi M, Hanson HL, Steinberg JP, Masopust D, Wherry EJ, Altman JD, Rouse BT, Freeman GJ, Ahmed R, Grakoui A. Liver-infiltrating lymphocytes in chronic human hepatitis C virus infection display an exhausted phenotype with high levels of PD-1 and low levels of CD127 expression. *J Virol* 2007; **81**: 2545-2553
 - 54 **Mescher MF**, Curtsinger JM, Agarwal P, Casey KA, Gerner M, Hammerbeck CD, Popescu F, Xiao Z. Signals required for programming effector and memory development by CD8+ T cells. *Immunol Rev* 2006; **211**: 81-92
 - 55 **Curtsinger JM**, Johnson CM, Mescher MF. CD8 T cell clonal expansion and development of effector function require prolonged exposure to antigen, costimulation, and signal 3 cytokine. *J Immunol* 2003; **171**: 5165-5171
 - 56 **Bigger CB**, Brasky KM, Lanford RE. DNA microarray analysis of chimpanzee liver during acute resolving hepatitis C virus infection. *J Virol* 2001; **75**: 7059-7066
 - 57 **Williams MA**, Bevan MJ. Effector and memory CTL differentiation. *Annu Rev Immunol* 2007; **25**: 171-192
 - 58 **Marzo AL**, Klonowski KD, Le Bon A, Borrow P, Tough DF, Lefrançois L. Initial T cell frequency dictates memory CD8+ T cell lineage commitment. *Nat Immunol* 2005; **6**: 793-799
 - 59 **Vezys V**, Masopust D, Kemball CC, Barber DL, O'Mara LA, Larsen CP, Pearson TC, Ahmed R, Lukacher AE. Continuous recruitment of naive T cells contributes to heterogeneity of antiviral CD8 T cells during persistent infection. *J Exp Med*

- 2006; **203**: 2263-2269
- 60 **Shin H**, Blackburn SD, Blattman JN, Wherry EJ. Viral antigen and extensive division maintain virus-specific CD8 T cells during chronic infection. *J Exp Med* 2007; **204**: 941-949
 - 61 **Fuller MJ**, Khanolkar A, Tebo AE, Zajac AJ. Maintenance, loss, and resurgence of T cell responses during acute, protracted, and chronic viral infections. *J Immunol* 2004; **172**: 4204-4214
 - 62 **Alves NL**, Hooibrink B, Arosa FA, van Lier RA. IL-15 induces antigen-independent expansion and differentiation of human naive CD8+ T cells *in vitro*. *Blood* 2003; **102**: 2541-2546
 - 63 **Maier H**, Isogawa M, Freeman GJ, Chisari FV. PD-1:PD-L1 interactions contribute to the functional suppression of virus-specific CD8+ T lymphocytes in the liver. *J Immunol* 2007; **178**: 2714-2720
 - 64 **van Faassen H**, Saldanha M, Gilbertson D, Dudani R, Krishnan L, Sad S. Reducing the stimulation of CD8+ T cells during infection with intracellular bacteria promotes differentiation primarily into a central (CD62LhighCD44high) subset. *J Immunol* 2005; **174**: 5341-5350
 - 65 **Tussey LG**, Nair US, Bachinsky M, Edwards BH, Bakari J, Grimm K, Joyce J, Vessey R, Steigbigel R, Robertson MN, Shiver JW, Goepfert PA. Antigen burden is major determinant of human immunodeficiency virus-specific CD8+ T cell maturation state: potential implications for therapeutic immunization. *J Infect Dis* 2003; **187**: 364-374
 - 66 **Papagno L**, Spina CA, Marchant A, Salio M, Rufer N, Little S, Dong T, Chesney G, Waters A, Easterbrook P, Dunbar PR, Shepherd D, Cerundolo V, Emery V, Griffiths P, Conlon C, McMichael AJ, Richman DD, Rowland-Jones SL, Appay V. Immune activation and CD8+ T-cell differentiation towards senescence in HIV-1 infection. *PLoS Biol* 2004; **2**: E20
 - 67 **Pulendran B**, Ahmed R. Translating innate immunity into immunological memory: implications for vaccine development. *Cell* 2006; **124**: 849-863
 - 68 **Meylan E**, Curran J, Hofmann K, Moradpour D, Binder M, Bartenschlager R, Tschopp J. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature* 2005; **437**: 1167-1172
 - 69 **Masopust D**, Vezys V, Marzo AL, Lefrançois L. Preferential localization of effector memory cells in nonlymphoid tissue. *Science* 2001; **291**: 2413-2417
 - 70 **Masopust D**, Vezys V, Wherry EJ, Barber DL, Ahmed R. Cutting edge: gut microenvironment promotes differentiation of a unique memory CD8 T cell population. *J Immunol* 2006; **176**: 2079-2083
 - 71 **Accapezzato D**, Francavilla V, Paroli M, Casciaro M, Chircu LV, Cividini A, Abrignani S, Mondelli MU, Barnaba V. Hepatic expansion of a virus-specific regulatory CD8(+) T cell population in chronic hepatitis C virus infection. *J Clin Invest* 2004; **113**: 963-972
 - 72 **He XS**, Rehermann B, Lopez-Labrador FX, Boisvert J, Cheung R, Mumm J, Wedemeyer H, Berenguer M, Wright TL, Davis MM, Greenberg HB. Quantitative analysis of hepatitis C virus-specific CD8(+) T cells in peripheral blood and liver using peptide-MHC tetramers. *Proc Natl Acad Sci USA* 1999; **96**: 5692-5697
 - 73 **Boni C**, Fiscaro P, Valdatta C, Amadei B, Di Vincenzo P, Giuberti T, Laccabue D, Zerbini A, Cavalli A, Missale G, Bertoletti A, Ferrari C. Characterization of hepatitis B virus (HBV)-specific T-cell dysfunction in chronic HBV infection. *J Virol* 2007; **81**: 4215-4225
 - 74 **Sprengers D**, van der Molen RG, Kusters JG, De Man RA, Niesters HG, Schalm SW, Janssen HL. Analysis of intrahepatic HBV-specific cytotoxic T-cells during and after acute HBV infection in humans. *J Hepatol* 2006; **45**: 182-189
 - 75 **de Bree GJ**, van Leeuwen EM, Out TA, Jansen HM, Jonkers RE, van Lier RA. Selective accumulation of differentiated CD8+ T cells specific for respiratory viruses in the human lung. *J Exp Med* 2005; **202**: 1433-1442
 - 76 **Hislop AD**, Kuo M, Drake-Lee AB, Akbar AN, Bergler W, Hammerschmitt N, Khan N, Palendira U, Leese AM, Timms JM, Bell AI, Buckley CD, Rickinson AB. Tonsillar homing of Epstein-Barr virus-specific CD8+ T cells and the virus-host balance. *J Clin Invest* 2005; **115**: 2546-2555
 - 77 **Shacklett BL**, Cox CA, Quigley MF, Kreis C, Stollman NH, Jacobson MA, Andersson J, Sandberg JK, Nixon DF. Abundant expression of granzyme A, but not perforin, in granules of CD8+ T cells in GALT: implications for immune control of HIV-1 infection. *J Immunol* 2004; **173**: 641-648
 - 78 **Wherry EJ**, Barber DL, Kaech SM, Blattman JN, Ahmed R. Antigen-independent memory CD8 T cells do not develop during chronic viral infection. *Proc Natl Acad Sci USA* 2004; **101**: 16004-16009

S- Editor Ma N L- Editor Rippe RA E- Editor Ma WH



TOPIC HIGHLIGHT

Robert Thimme, MD, Professor, Series Editor

Regulatory T cells in viral hepatitis

Eva Billerbeck, Tobias Böttler, Robert Thimme

Eva Billerbeck, Tobias Böttler, Robert Thimme, Department of Medicine II, University Hospital Freiburg, Germany
Correspondence to: Robert Thimme, MD, Department of Medicine II, University Hospital Freiburg, Hugstetter Strasse 55, D-79106 Freiburg, Germany. thimme@med1.ukl.uni-freiburg.de
Telephone: +49-761-2703280 Fax: +49-761-2703372
Received: June 26, 2007 Revised: July 9, 2007

Abstract

The pathogenesis and outcome of viral infections are significantly influenced by the host immune response. The immune system is able to eliminate many viruses in the acute phase of infection. However, some viruses, like hepatitis C virus (HCV) and hepatitis B virus (HBV), can evade the host immune responses and establish a persistent infection. HCV and HBV persistence is caused by various mechanisms, like subversion of innate immune responses by viral factors, the emergence of T cell escape mutations, or T cell dysfunction and suppression. Recently, it has become evident that regulatory T cells may contribute to the pathogenesis and outcome of viral infections by suppressing antiviral immune responses. Indeed, the control of HCV and HBV specific immune responses mediated by regulatory T cells may be one mechanism that favors viral persistence, but it may also prevent the host from overwhelming T cell activity and liver damage. This review will focus on the role of regulatory T cells in viral hepatitis.

© 2007 WJG. All rights reserved.

Key words: Regulatory T cells; Viral hepatitis; Immuno-regulation

Billerbeck E, Böttler T, Thimme R. Regulatory T cells in viral hepatitis. *World J Gastroenterol* 2007; 13(36): 4858-4864

<http://www.wjgnet.com/1007-9327/13/4858.asp>

INTRODUCTION

An infection with HCV or HBV activates the immune system to defend the host with a broad range of innate and adaptive immune responses. Macrophages, natural killer cells and neutrophils are an important part of the innate immune response that produces inflammatory and antiviral cytokines. Activated dendritic cells induce the differentiation of naïve T cells into virus specific CD4+

and CD8+ T cells for adaptive immunity. CD4+ T cells Type 2 induce B cells to produce antiviral antibodies; CD4+ T cells Type 1 cells secrete IFN- γ and activate the massive proliferation of cytotoxic CD8+ cells that destroy infected cells or secrete proinflammatory cytokines^[1-3].

These complex molecular and cellular mechanisms help to control and, in the best case, to eliminate the virus in the acute phase of infection. However, in the majority of HCV and a significant amount of HBV infections the immune system fails to eliminate the virus and viral persistence is established. Although essential for successful virus elimination, the virus specific T cell responses of the host may also cause tissue damage and autoimmune reactions in the liver, especially in the setting of chronic viral infection. Therefore, many regulatory mechanisms of the immune system control the virus specific immune responses in order to prevent massive tissue damage or autoimmune disease.

Over the last few years, it has become evident that regulatory T cells (T_{reg} cells) may play an important role in the suppression of virus specific immune responses^[4-6]. Indeed, several studies suggest a role of diverse populations of T_{reg} cells in the natural course of HCV and HBV infections. In this review, we will summarize the current knowledge about the role of regulatory T cells in HCV and HBV infection.

REGULATORY T CELL SUBSETS

A regulatory phenotype of a T cell population was first described for a CD4+ T cell subset that constitutively expresses the interleukin 2 receptor α -chain (CD25). In 1995 Sakaguchi *et al* showed that the transfer of lymphocytes depleted of CD4+CD25+ T cells into athymic mice caused the development of various autoimmune diseases in the recipient mice. Interestingly, the reconstitution with CD4+CD25+ T cells prevented autoimmune reactions in these mice, indicating a function of this T cell subpopulation in the control of self tolerance^[7]. In the last decade numerous studies in mice and men showed that diverse T cell populations exhibit regulatory capacity and play an important role in the suppression of immune responses to self as well as foreign antigens^[8]. Regulatory T cells are divided into a natural CD4+CD25+ T_{reg} cell population and diverse populations of induced or adaptive T_{reg} cells^[9]. Natural T_{reg} cells develop in the thymus under strong TCR engagement with self peptides and play an important role in the maintenance of self-tolerance and immune homeostasis.

About 5%-10% of CD4⁺ T cells in mice and humans are natural T_{reg} cells^[10]. Natural CD4⁺CD25⁺ T_{reg} cells constitutively express cytotoxic T-lymphocyte antigen 4 (CTLA4), glucocorticoid-induced TNF receptor family related protein (GITR)^[11,12] and the forkhead family transcription factor FoxP3^[13,14]. Of note, mutations in the gene *foxP3* cause the absence of natural T_{reg} cells and a loss of self tolerance. Indeed, it has been shown that FoxP3 expression is the essential factor for the induction of the natural T_{reg} cell population. FoxP3 is the best marker for the identification of natural T_{reg} cells in mice and men, thus far^[15]. However, it has recently been shown that FoxP3 may also be transiently induced in activated human T cells^[16,17].

In contrast to natural T_{regs} cells, induced T_{regs} cells develop from non regulatory T cells in the periphery and not in the thymus. Diverse populations of induced regulatory T cells have been identified, thus far. Among those, CD4⁺ cells secreting IL-10 or TGF- β , named TR1- or TH3 cells, as well as CD4⁺CD25⁺FoxP3⁺ T cells and CD8⁺ T cells with various phenotypes have been shown to have a regulatory capacity^[8,18]. The extrathymically conversion of non-regulatory T cells into regulatory T cells requires special immunological conditions. In many respects, the cytokine TGF- β and a distinct mode of antigen exposure have been shown to play an important role in the development of induced T_{reg} cells^[8,19]. Indeed, several studies in both mice and humans demonstrated that naïve and memory CD4⁺ T cells can be converted into CD4⁺CD25⁺FoxP3⁺ regulatory T cells in the presence of TGF- β ^[20-23]. In addition, it has been shown in several mouse models that a specific way of antigen presentation, e.g. continuous exposure to low dose antigen or to a systemic peripheral antigen, can lead to the expansion of induced CD4⁺CD25⁺FoxP3⁺ regulatory T cells^[24-26].

Overall, these results and the phenotypical diversity of induced T_{reg} cell subpopulations indicate that several different mechanisms of T_{reg} cell development may exist in the periphery that still need to be defined for each subpopulation of induced T_{reg} cells. Furthermore, lineage relationship and functional overlap of induced T_{reg} cells and natural T_{reg} cells still need to be characterized in detail.

FUNCTIONAL CHARACTERISTICS OF REGULATORY T CELLS

The major function of natural and induced T_{reg} cells is the suppression of immune responses to self or foreign antigens. Indeed, numerous studies in mice and humans showed that regulatory T cells suppress the proliferation, cytokine-production (IFN- γ , IL-2) and cytolytic activity of naïve and antigen specific CD4⁺ and CD8⁺ cells. In addition, T_{reg} cells are able to suppress the functions of antigen presenting cells and B cells^[27]. T_{reg} cells may mediate their suppressive activity either through the secretion of anti-inflammatory cytokines like IL-10 or TGF- β , direct killing of the target cells or distinct cell-cell contact dependent mechanisms^[27]. The surface molecules CTLA4 and GITR have been suggested to play a role in direct cell-cell contact mediated suppression. CTLA-4

expressed on T_{reg} cells may bind to CD80/CD86 expressed on antigen presenting cells to activate the IDO (indoleamine 2,3-dioxygenase) dependent generation of tryptophan. A decrease of free tryptophan reduces T cell activation^[28,29]. The transfer of cAMP from T_{reg} to effector cells via gap junction contact has recently been proposed to be another possible mode of cell-cell contact mediated suppression. cAMP inhibits the proliferation and IL-2 secretion of T cells^[30]. In addition, T_{reg} cells have also been shown to generate extracellular adenosine that suppresses T cells responses^[31]. However, the mechanisms and the antigen-specificity of T_{reg} cell mediated immunosuppression are still largely unknown.

In addition to the ability to control immune responses, at least those T_{reg} cell populations that express FoxP3 share other functional characteristics that distinguish them from effector T cells. Indeed, the expression of the transcription factor FoxP3 significantly influences the phenotype and function of T_{reg} cells^[32]. FoxP3⁺ T cells show different TCR signaling patterns from effector T cells, do not proliferate well, when cultured *in vitro* and do not produce IL-2 or other inflammatory cytokines^[32]. Recently, genome wide analysis of FoxP3 target genes in mouse natural T_{reg} cells revealed that FoxP3 regulates those genes involved into TCR signaling pathways and cytokine-production as well as genes encoding for T_{reg} cell associated surface molecules like CD25 or GITR^[33,34]. In conclusion, the functional as well as the phenotypical differences between T_{reg} cells and effector T cells are largely dependent on the expression of FoxP3 in regulatory T cells.

REGULATORY T CELLS IN VIRAL INFECTION

Many viruses, like HCV and HBV, are able to evade the host immune response and to establish chronic infection. Efficient virus specific T cell responses are critical to eliminate the virus in the acute phase of infection. Importantly however, viruses have evolved strategies of immune evasion to subvert innate and adaptive immune responses and to facilitate viral persistence^[35].

Growing evidence suggests that regulatory T cells may play an important role in the suppression of antiviral T cell responses in the acute and chronic phase of infection. Indeed, the virus specific induction of regulatory T cells may have two very different consequences: first, it may help the virus to establish viral persistence and second, it may be an important process that occurs to prevent excessive immunopathological damage^[4,5].

First evidence that regulatory T cells may play a role in viral infection was forthcoming from a study by Souvas *et al* who showed that the depletion of CD4⁺CD25⁺ T cells in mice infected with herpes simplex virus (HSV) enhanced virus specific CD8⁺ T cell activity in the acute phase of infection as well as after viral clearance. Furthermore, HSV infection appeared to have a direct effect on natural T_{reg} cells, since regulatory T cells from HSV infected mice showed an increased suppressive capacity towards HSV specific and unspecific CD8⁺ T cell responses^[36]. While these findings indicate a detrimental role of natural T_{reg} in the host-virus immune balance, another study with HSV infected mice proposed a

protective role of natural T_{reg} cells in viral infection. Mice suffering from blinding keratitis, caused by HSV infection, showed much more severe lesions in the eyes when depleted of natural CD4+CD25+ regulatory T cells^[37].

Regulatory T cells have also been shown to play a role in chronic retroviral infections, indicated by studies with mice infected with Friend virus (FV). CD8+ T cells are critical for clearance of FV in the acute phase of infection, while insufficient CD8+ T cell effector functions are associated with viral persistence. A study by Dittmer *et al* showed that immunosuppression and CD8+ T cell impairment in chronic FV infection is a result of induced T_{reg} cell activity. Indeed, adoptive transfer experiments revealed that IL-10 secreting CD4+ cells from persistently infected mice suppressed antigen specific IFN- γ secretion of CD8+ cells^[38]. Furthermore, a kinetic analysis of T cell responses in acute FV infection demonstrated that the onset CD8+ T cell dysfunction as early as 2 wk after infection was associated with the expansion of induced T_{reg} cells^[39].

Taken together, these *in vivo* results from different mouse models suggest the important role of regulatory T cells in the suppression of virus specific T cell responses. In the following, recent human studies indicating the role of regulatory T cells in viral hepatitis will be discussed in more detail.

REGULATORY T CELLS IN HCV INFECTION

About 170 million people worldwide suffer from chronic hepatitis C virus infection. HCV is able to persist in up to 70% of those infected and may cause chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. HCV is a positive-stranded RNA virus that belongs to the Flaviviruses^[1].

HCV clearance is associated with a vigorous HCV specific CD4+ and CD8+ T cell response in the acute phase of infection. In contrast, viral persistence is associated with a weak and dysfunctional virus specific T cell response^[40]. Several possible mechanisms of T cell failure and HCV immune evasion have been proposed, like T cell deletion, T cell dysfunction and the emergence of viral escape mutations^[2,41]. Recently, the possible role of different regulatory T cell populations in HCV persistence has also been suggested.

Indeed, several groups have shown a higher frequency of CD4+CD25+ regulatory T cells in the blood of chronically HCV infected persons versus recovered or healthy persons^[42-45] and the presence of CD4+FoxP3+ T cells in the liver of chronically HCV infected patients^[45,46]. However, whether these cells are natural T_{reg} cells that originate from the thymus or whether these cells are induced T_{regs} that developed from conventional CD4+ T cells upon HCV antigen contact in the periphery, remains to be determined. Of note, CD4+CD25+ regulatory T cells from chronically HCV infected patients are capable of suppressing HCV specific CD8+ T cell and CD4+ T cell proliferation as well as CD8+ T cell IFN- γ secretion in a dose-dependent manner that requires direct cell-cell contact. However, CD4+CD25+ T_{reg} cells of infected subjects did not only suppress HCV specific T

cell proliferation, but also Influenza-, CMV-, and EBV specific T cell responses, suggesting an antigen unspecific inhibition of CD8+ T cells^[42,45].

The role of immunosuppressive cytokines, such as IL-10 and TGF- β , in the CD4+CD25+ T_{reg} cells dependent suppression remains controversial. Some studies reported that T_{reg} cells secrete IL-10 and TGF- β after HCV antigen stimulation and that TGF- β neutralization reverses T_{reg} cell mediated suppression of virus specific T cell responses^[43,47], while others did not observe this effect^[42,45].

It is important to note that CD4+CD25+ T_{reg} cells obtained from chronically HCV infected patients had an increased suppressive activity against HCV specific CD8+ T cells compared to natural T_{reg} cells isolated from subjects that had recovered from acute HCV infection, suggesting that chronic HCV infection leads to the expansion and activation of CD4+CD25+ T_{reg} cells. However the suppressive effect observed in patients who successfully cleared the virus was still significant^[42]. Furthermore, a recent study with HCV infected chimpanzees, the only animal model for HCV infection, showed that the frequency of CD4+CD25+FoxP3+ T_{reg} cells and the suppressive capacity of those cells against virus specific T cell responses were as high in HCV recovered chimpanzees as in persistently HCV infected chimpanzees^[48]. These results suggest that CD4+CD25+ T_{reg} cells do not only suppress virus specific T cell responses in chronic infection but may also control memory T cells after virus recovery. Of note, further analysis of CD4+CD25+ T_{reg} cells in chimpanzees revealed that T_{reg} cells from chronically HCV infected and recovered chimpanzees displayed fewer T cell receptor excision cycles and a better proliferative capacity *in vitro* compared to T_{reg} cells from HCV naïve chimpanzees. These data may indicate HCV specific proliferation of T_{reg} cells in HCV infected chimpanzees.

First evidence that T_{reg} cells may be induced by HCV antigens was provided by a study that examined the CD4+ T cell response to the HCV core protein. Interestingly, HCV specific IL-10 secreting T cells were detected in the blood of chronic HCV infected persons^[49]. These regulatory Tr1 cells recognized the same epitopes on the core protein as IFN- γ producing T_H1 cells. However, HCV specific IL-10 secretion is not limited to the CD4+ T cell subset, since IL-10 producing HCV specific CD8+ T cells were identified that suppressed IFN- γ secretion of CD8+ T cells targeting the same epitope as the IL-10 secreting cells^[50]. Of note, both cell subsets were predominantly present in the liver, suggesting a compartmentalization of effector and regulatory T cells to the site of infection. In addition, another study also reported the accumulation of HCV specific IL-10 producing CD8+ T cells in the liver of chronically infected patients. *In situ* staining of liver biopsies revealed that IL-10+ HCV specific CD8+ T cells are located in liver areas with low hepatocellular apoptosis and low liver fibrosis, further supporting a potential role of these cells in the prevention of liver damage^[51]. However, these studies did not determine the FoxP3 expression of these CD8+ T cells.

The conclusion, that regulatory CD8+ T cells may play an important role in chronic HCV infection is further underlined by the observation that HCV specific CD8+

Table 1 Regulatory T cell subsets in HCV and HBV infection

Regulatory T cell phenotype	Virus	Compartment	Suppression	Cytokines
CD4+CD25+ (FoxP3+) ^[42-46,48,54,56,58,61,62]	HCV	Blood/Liver	Cell-cell contact dependent suppression of antigen specific and unspecific T cell proliferation and cytokine production	In part IL-10/ TGF-β
CD4+ (T _H 1 cells) ^[49]	HCV	Blood		IL-10
CD8+ (FoxP3+) ^[50-52]	HCV	Blood/Liver	Suppression of antigen specific T cell proliferation and INF-γ secretion	IL-10/TGF-β
CD8+FoxP3+ CTLA-4+GITR ⁺ ^[53]	HCV	Blood	Cell-cell contact suppression of antigen unspecific T cell proliferation	IFN-γ

CD25⁺FoxP3⁺ T cells from the blood of chronically infected patients suppress HCV specific T cell responses *via* TGF-β secretion. Of note, the blockade of TGF-β markedly enhanced the HCV specific IFN-γ secretion by CD4⁺ and CD8⁺ T cells^[52].

A population of HCV specific FoxP3⁺CD8⁺ T cells that suppressed CD4⁺ and CD8⁺ T cell proliferation in a cell-cell contact dependent manner has also been described^[53]. Indeed, these cells expanded simultaneously with FoxP3⁺CD8⁺ effector T cells after *in vitro* HCV specific peptide stimulation of peripheral blood mononuclear cells (PBMC) from chronically HCV infected patients. These results suggest that stimulation with a defined viral antigen leads to the expansion of two distinct CD8⁺ T cell populations: FoxP3⁺ effector as well as FoxP3⁺ regulatory T cells. FoxP3 expression on virus-specific CD8⁺ T cells *ex vivo* has not been shown, however. Of note, *in vitro* stimulation of PBMC from chronically HCV infected patients with HCV specific antigens also resulted in an expansion of HCV specific CD4⁺CD25⁺FoxP3⁺ regulatory T cells^[54]. However, at least for the CD8⁺ T cell compartment this is not a HCV specific effect since the expansion of virus specific FoxP3⁺ regulatory CD8⁺ T cells after *in vitro* peptide stimulation was also detected in influenza specific CD8⁺ T cells.

In summary, all these studies suggest a role of different regulatory T cell populations in the pathogenesis of HCV infection (Table 1). The elevated frequency of CD4⁺CD25⁺ regulatory T cells in the blood of chronically HCV infected patients, the ability of these cells to suppress HCV specific T cell responses, the accumulation of FoxP3⁺ T cells in the liver as well as the existence of different HCV specific regulatory T cell populations, strongly indicate that HCV infection induces virus-specific regulatory T cells that may contribute to viral persistence by suppressing HCV specific T cell responses. The presence of T_{reg} cells, especially in the liver, may also protect the host from tissue damage. In this context, it is interesting to note, that one study showed that HCV infected patients with normal alanine transaminase (ALT) levels have an increased frequency of HCV specific TGF-β secreting CD4⁺CD25⁺ regulatory T cells combined with decreased liver inflammation compared to patients with elevated ALT levels^[47].

REGULATORY T CELLS IN HBV INFECTION

Hepatitis B virus is a hepatotropic DNA virus infecting

about 300 million people worldwide. About 5%-10% of acutely infected patients develop a persistent HBV infection that is associated with T cell hyporesponsiveness and dysfunctions^[55]. Thus, T_{reg} cells may also play a role in HBV infection. Indeed, one study reported a high frequency of CD4⁺ CD25⁺ T_{reg} cells in the blood of chronically HBV infected subjects and an increase of HBV specific T cell proliferation after depletion of CD4⁺CD25⁺ T cells^[56]. Although these data suggest a potential role of T_{reg} cells in mediating T cell dysfunction during chronic HBV infection, another study reported discrepant results^[57]. In fact, neither a higher frequency nor an elevated suppressive capacity of CD4⁺CD25⁺ T_{reg} cells isolated from the blood of chronically infected patients compared to CD4⁺CD25⁺ T_{reg} cells from persons recovered from HBV infection, were observed in this study^[57].

However, recent studies shed more light on these controversial results about the role of T_{reg} cells in HBV infection. Indeed, a broad analysis of the frequency and function of CD4⁺CD25⁺ T_{reg} cells in the blood and liver of patients with acute HBV, chronic HBV, chronic severe HBV and healthy controls revealed that only patients with a chronic severe HBV infection showed a significant higher level of CD4⁺CD25⁺ T_{reg} cells in the blood compared to patients with mild course of chronic HCV and acute HCV infection^[58]. A significant accumulation of CD4⁺CD25⁺FoxP3⁺ T_{reg} cells in the liver was found in patients with chronic HBV and chronic severe HBV infection^[58,59]. A positive correlation between HBeAg level, HBV DNA level and the frequency of CD4⁺CD25⁺ T_{reg} cells in the blood of chronically infected patients further supports the role of T_{reg} cells in HBV infection^[58-60]. Finally, two studies showed the presence of HBeAg-specific CD4⁺CD25⁺FoxP3⁺ T_{reg} cells in chronically HBV infected patients^[61,62]. Interestingly, HBeAg specific CD4⁺CD25⁺ T_{reg} cells declined in the blood of patients during acute exacerbation of hepatitis in the immunoactive phase in chronic HBV infection while the HBeAg specific CD8⁺ T cell frequency increased at the same time. These data indicate that HBV specific T_{reg} cells may suppress HBV specific CD8⁺ T cells responses during flares in chronic HBV infection and may thus contribute to protection from severe hepatitis^[61].

The analysis of T_{reg} cells during acute HBV infection showed that T_{reg} cells may also play a role in this state of the infection. Indeed, the frequency of CD4⁺CD25⁺ T_{reg} cells was normal in the early acute phase of infection, increased during the convalescent phase and decreased

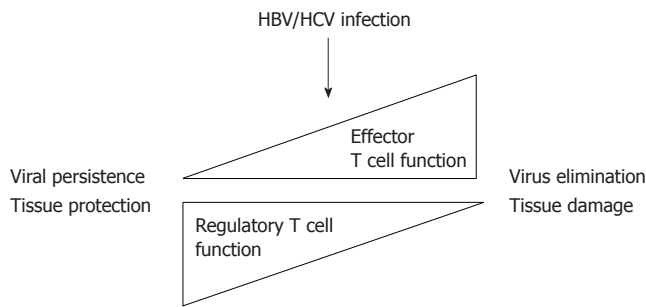


Figure 1 Regulatory T cells may contribute to viral persistence but they may also prevent tissue damage.

again to normal after virus resolution^[58]. Of note, CD4+CD25+ T_{reg} cells isolated from the blood of acutely HBV infected patients had an increased suppressive activity against HBV specific T cell responses compared to antigen unspecific T cell responses, suggesting the generation of HBV antigen specific T_{reg} cells during acute HBV infection^[58]. In summary, as in HCV infection, recent studies suggest that regulatory T cells do also play a role in HBV infection (Table 1).

FUTURE PERSPECTIVES

Taken together, there is strong evidence that different regulatory T cell populations mediate virus-specific T cell suppression in HCV and HBV infection. This immune suppression may contribute to viral persistence, but also to protection from overwhelming liver damage (Figure 1). Although, the studies discussed in this review give important first insights into the role of regulatory T cells in viral hepatitis, they also raise many questions. In fact, HCV infection in particular seems to induce a striking number of distinct HCV specific regulatory T cell populations. However, the lineage relationship between these T_{reg} cell populations as well as their relationship with HCV specific effector T cells remains elusive. It is unclear whether the CD4+CD25+ T_{reg} cells analyzed in HCV infected patients natural T_{reg} cells derive from the thymus develop from CD4+ T cells upon HCV antigen encounter in the periphery. The latter is most likely for HCV specific regulatory CD8+ T cells. However, if HCV specific T_{reg} cells are induced in the periphery, do they develop from naïve T cells as distinct induced CD4+ or CD8+ regulatory T cell lineages or do they generate from HCV specific effector memory T cells in a certain immunological context of the virus infection?

Other important questions that need to be addressed are the place and time point of regulatory T cell induction in HCV and HBV infection, as well as the mode and antigen specificity of T_{reg} cell mediated suppression in both viral infections. In addition, the presence and action of regulatory T cells in the liver, the site of virus replication and chronic inflammation should be analyzed in more detail. Furthermore, it will be important to determine if virus specific regulatory T cells do only play a role in the progression of a chronic infection or if they are already activated during the acute phase of infection and contribute to the development of viral persistence, as has

already been indicated in the case of HBV infection.

Of note, T_{reg} cells may serve as a potential target for therapeutic interventions. The depletion of regulatory T cells during an acute viral infection may have the therapeutic potential to prevent viral persistence. Furthermore, a manipulation of regulatory T cells may also improve vaccine efficiency. Studies with mouse models already suggest that depletion of CD4+ CD25+ T cells can help to resolve an infection^[36] or to enhance the effect of a virus DNA vaccination^[63,64]. However, a depletion of regulatory T cells in the setting of a chronic viral infection could also lead to massive tissue damage, if virus specific CD8+ T cells are no longer suppressed. Therefore, an application of these approaches in humans requires a more detailed knowledge about the exact interplay of regulatory T cells, viruses and virus specific immune responses. Otherwise, the overall function of regulatory T cells in the maintenance of immune homeostasis and self tolerance could be dangerously disturbed.

REFERENCES

- 1 Dustin LB, Rice CM. Flying under the radar: the immunobiology of hepatitis C. *Annu Rev Immunol* 2007; **25**: 71-99
- 2 Bowen DG, Walker CM. Adaptive immune responses in acute and chronic hepatitis C virus infection. *Nature* 2005; **436**: 946-952
- 3 Wieland SF, Chisari FV. Stealth and cunning: hepatitis B and hepatitis C viruses. *J Virol* 2005; **79**: 9369-9380
- 4 Belkaid Y, Rouse BT. Natural regulatory T cells in infectious disease. *Nat Immunol* 2005; **6**: 353-360
- 5 Mills KH. Regulatory T cells: friend or foe in immunity to infection? *Nat Rev Immunol* 2004; **4**: 841-855
- 6 Suvas S, Rouse BT. Treg control of antimicrobial T cell responses. *Curr Opin Immunol* 2006; **18**: 344-348
- 7 Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 1995; **155**: 1151-1164
- 8 Shevach EM. From vanilla to 28 flavors: multiple varieties of T regulatory cells. *Immunity* 2006; **25**: 195-201
- 9 Bluestone JA, Abbas AK. Natural versus adaptive regulatory T cells. *Nat Rev Immunol* 2003; **3**: 253-257
- 10 Sakaguchi S. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol* 2005; **6**: 345-352
- 11 Shimizu J, Yamazaki S, Takahashi T, Ishida Y, Sakaguchi S. Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. *Nat Immunol* 2002; **3**: 135-142
- 12 Read S, Malmström V, Powrie F. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. *J Exp Med* 2000; **192**: 295-302
- 13 Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 2003; **4**: 330-336
- 14 Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003; **299**: 1057-1061
- 15 Fontenot JD, Rasmussen JP, Williams LM, Dooley JL, Farr AG, Rudensky AY. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity* 2005; **22**: 329-341
- 16 Gavin MA, Torgerson TR, Houston E, DeRoos P, Ho WY, Stray-Pedersen A, Ocheltree EL, Greenberg PD, Ochs HD, Rudensky AY. Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell

- development. *Proc Natl Acad Sci USA* 2006; **103**: 6659-6664
- 17 **Wang J**, Ioan-Facsinay A, van der Voort EI, Huizinga TW, Toes RE. Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. *Eur J Immunol* 2007; **37**: 129-138
 - 18 **Fehérvári Z**, Sakaguchi S. CD4+ Tregs and immune control. *J Clin Invest* 2004; **114**: 1209-1217
 - 19 **Akbar AN**, Vukmanovic-Stejic M, Taams LS, Macallan DC. The dynamic co-evolution of memory and regulatory CD4+ T cells in the periphery. *Nat Rev Immunol* 2007; **7**: 231-237
 - 20 **Chen W**, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, McGrady G, Wahl SM. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* 2003; **198**: 1875-1886
 - 21 **Zheng SG**, Gray JD, Ohtsuka K, Yamagiwa S, Horwitz DA. Generation ex vivo of TGF-beta-producing regulatory T cells from CD4+CD25- precursors. *J Immunol* 2002; **169**: 4183-4189
 - 22 **Yamagiwa S**, Gray JD, Hashimoto S, Horwitz DA. A role for TGF-beta in the generation and expansion of CD4+CD25+ regulatory T cells from human peripheral blood. *J Immunol* 2001; **166**: 7282-7289
 - 23 **Davidson TS**, DiPaolo RJ, Andersson J, Shevach EM. Cutting Edge: IL-2 is essential for TGF-beta-mediated induction of Foxp3+ T regulatory cells. *J Immunol* 2007; **178**: 4022-4026
 - 24 **Kretschmer K**, Apostolou I, Hawiger D, Khazaie K, Nussenzweig MC, von Boehmer H. Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol* 2005; **6**: 1219-1227
 - 25 **Knoechel B**, Lohr J, Kahn E, Bluestone JA, Abbas AK. Sequential development of interleukin 2-dependent effector and regulatory T cells in response to endogenous systemic antigen. *J Exp Med* 2005; **202**: 1375-1386
 - 26 **Apostolou I**, von Boehmer H. *In vivo* instruction of suppressor commitment in naive T cells. *J Exp Med* 2004; **199**: 1401-1408
 - 27 **von Boehmer H**. Mechanisms of suppression by suppressor T cells. *Nat Immunol* 2005; **6**: 338-344
 - 28 **Mellor AL**, Munn DH. IDO expression by dendritic cells: tolerance and tryptophan catabolism. *Nat Rev Immunol* 2004; **4**: 762-774
 - 29 **Fallarino F**, Grohmann U, Hwang KW, Orabona C, Vacca C, Bianchi R, Belladonna ML, Fioretti MC, Alegre ML, Puccetti P. Modulation of tryptophan catabolism by regulatory T cells. *Nat Immunol* 2003; **4**: 1206-1212
 - 30 **Bopp T**, Becker C, Klein M, Klein-Hessling S, Palmetshofer A, Serfling E, Heib V, Becker M, Kubach J, Schmitt S, Stoll S, Schild H, Staeger MS, Stassen M, Jonuleit H, Schmitt E. Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression. *J Exp Med* 2007; **204**: 1303-1310
 - 31 **Deaglio S**, Dwyer KM, Gao W, Friedman D, Usheva A, Erat A, Chen JF, Enjyoji K, Linden J, Oukka M, Kuchroo VK, Strom TB, Robson SC. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J Exp Med* 2007; **204**: 1257-1265
 - 32 **Campbell DJ**, Ziegler SF. FOXP3 modifies the phenotypic and functional properties of regulatory T cells. *Nat Rev Immunol* 2007; **7**: 305-310
 - 33 **Marson A**, Kretschmer K, Frampton GM, Jacobsen ES, Polansky JK, MacIsaac KD, Levine SS, Fraenkel E, von Boehmer H, Young RA. Foxp3 occupancy and regulation of key target genes during T-cell stimulation. *Nature* 2007; **445**: 931-935
 - 34 **Zheng Y**, Josefowicz SZ, Kas A, Chu TT, Gavin MA, Rudensky AY. Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T cells. *Nature* 2007; **445**: 936-940
 - 35 **Klenerman P**, Hill A. T cells and viral persistence: lessons from diverse infections. *Nat Immunol* 2005; **6**: 873-879
 - 36 **Suvas S**, Kumaraguru U, Pack CD, Lee S, Rouse BT. CD4+CD25+ T cells regulate virus-specific primary and memory CD8+ T cell responses. *J Exp Med* 2003; **198**: 889-901
 - 37 **Suvas S**, Azkur AK, Kim BS, Kumaraguru U, Rouse BT. CD4+CD25+ regulatory T cells control the severity of viral immunoinflammatory lesions. *J Immunol* 2004; **172**: 4123-4132
 - 38 **Dittmer U**, He H, Messer RJ, Schimmer S, Olbrich AR, Ohlen C, Greenberg PD, Stromnes IM, Iwashiro M, Sakaguchi S, Evans LH, Peterson KE, Yang G, Hasenkrug KJ. Functional impairment of CD8(+) T cells by regulatory T cells during persistent retroviral infection. *Immunity* 2004; **20**: 293-303
 - 39 **Zelinskyy G**, Kraft AR, Schimmer S, Arndt T, Dittmer U. Kinetics of CD8+ effector T cell responses and induced CD4+ regulatory T cell responses during Friend retrovirus infection. *Eur J Immunol* 2006; **36**: 2658-2670
 - 40 **Shoukry NH**, Cawthon AG, Walker CM. Cell-mediated immunity and the outcome of hepatitis C virus infection. *Annu Rev Microbiol* 2004; **58**: 391-424
 - 41 **Thimme R**, Lohmann V, Weber F. A target on the move: innate and adaptive immune escape strategies of hepatitis C virus. *Antiviral Res* 2006; **69**: 129-141
 - 42 **Boettler T**, Spangenberg HC, Neumann-Haefelin C, Panther E, Urbani S, Ferrari C, Blum HE, von Weizsäcker F, Thimme R. T cells with a CD4+CD25+ regulatory phenotype suppress in vitro proliferation of virus-specific CD8+ T cells during chronic hepatitis C virus infection. *J Virol* 2005; **79**: 7860-7867
 - 43 **Cabrera R**, Tu Z, Xu Y, Firpi RJ, Rosen HR, Liu C, Nelson DR. An immunomodulatory role for CD4(+)CD25(+) regulatory T lymphocytes in hepatitis C virus infection. *Hepatology* 2004; **40**: 1062-1071
 - 44 **Sugimoto K**, Ikeda F, Stadanlick J, Nunes FA, Alter HJ, Chang KM. Suppression of HCV-specific T cells without differential hierarchy demonstrated ex vivo in persistent HCV infection. *Hepatology* 2003; **38**: 1437-1448
 - 45 **Rushbrook SM**, Ward SM, Unitt E, Vowler SL, Lucas M, Klenerman P, Alexander GJ. Regulatory T cells suppress in vitro proliferation of virus-specific CD8+ T cells during persistent hepatitis C virus infection. *J Virol* 2005; **79**: 7852-7859
 - 46 **Ward SM**, Fox BC, Brown PJ, Worthington J, Fox SB, Chapman RW, Fleming KA, Banham AH, Klenerman P. Quantification and localisation of FOXP3+ T lymphocytes and relation to hepatic inflammation during chronic HCV infection. *J Hepatol* 2007; **47**: 316-324
 - 47 **Bolacchi F**, Sinistro A, Ciaprinì C, Demin F, Capozzi M, Carducci FC, Drapeau CM, Rocchi G, Bergamini A. Increased hepatitis C virus (HCV)-specific CD4+CD25+ regulatory T lymphocytes and reduced HCV-specific CD4+ T cell response in HCV-infected patients with normal versus abnormal alanine aminotransferase levels. *Clin Exp Immunol* 2006; **144**: 188-196
 - 48 **Manigold T**, Shin EC, Mizukoshi E, Mihalik K, Murthy KK, Rice CM, Piccirillo CA, Rehermann B. Foxp3+CD4+CD25+ T cells control virus-specific memory T cells in chimpanzees that recovered from hepatitis C. *Blood* 2006; **107**: 4424-4432
 - 49 **MacDonald AJ**, Duffy M, Brady MT, McKiernan S, Hall W, Hegarty J, Curry M, Mills KH. CD4 T helper type 1 and regulatory T cells induced against the same epitopes on the core protein in hepatitis C virus-infected persons. *J Infect Dis* 2002; **185**: 720-727
 - 50 **Accapezzato D**, Francavilla V, Paroli M, Casciaro M, Chircu LV, Cividini A, Abrignani S, Mondelli MU, Barnaba V. Hepatic expansion of a virus-specific regulatory CD8(+) T cell population in chronic hepatitis C virus infection. *J Clin Invest* 2004; **113**: 963-972
 - 51 **Abel M**, Sène D, Pol S, Bourlière M, Poynard T, Charlotte F, Cacoub P, Caillat-Zucman S. Intrahepatic virus-specific IL-10-producing CD8 T cells prevent liver damage during chronic hepatitis C virus infection. *Hepatology* 2006; **44**: 1607-1616
 - 52 **Alatrakchi N**, Graham CS, van der Vliet HJ, Sherman KE, Exley MA, Koziel MJ. Hepatitis C virus (HCV)-specific CD8+ cells produce transforming growth factor beta that can suppress HCV-specific T-cell responses. *J Virol* 2007; **81**: 5882-5892
 - 53 **Billerbeck E**, Blum HE, Thimme R. Parallel expansion of human virus-specific FoxP3- effector memory and de novo-generated FoxP3+ regulatory CD8+ T cells upon antigen recognition in vitro. *J Immunol* 2007; **179**: 1039-1048
 - 54 **Li S**, Jones KL, Woollard DJ, Dromey J, Paukovics G, Plebanski M, Gowans EJ. Defining target antigens for CD25+ FOXP3+ IFN-gamma- regulatory T cells in chronic hepatitis C virus infection. *Immunol Cell Biol* 2007; **85**: 197-204
 - 55 **Rehermann B**, Nascimbeni M. Immunology of hepatitis B virus and hepatitis C virus infection. *Nat Rev Immunol* 2005; **5**: 215-229

- 56 **Stoop JN**, van der Molen RG, Baan CC, van der Laan LJ, Kuipers EJ, Kusters JG, Janssen HL. Regulatory T cells contribute to the impaired immune response in patients with chronic hepatitis B virus infection. *Hepatology* 2005; **41**: 771-778
- 57 **Franzese O**, Kennedy PT, Gehring AJ, Gotto J, Williams R, Maini MK, Bertolotti A. Modulation of the CD8+ T-cell response by CD4+ CD25+ regulatory T cells in patients with hepatitis B virus infection. *J Virol* 2005; **79**: 3322-3328
- 58 **Xu D**, Fu J, Jin L, Zhang H, Zhou C, Zou Z, Zhao JM, Zhang B, Shi M, Ding X, Tang Z, Fu YX, Wang FS. Circulating and liver resident CD4+CD25+ regulatory T cells actively influence the antiviral immune response and disease progression in patients with hepatitis B. *J Immunol* 2006; **177**: 739-747
- 59 **Yang G**, Liu A, Xie Q, Guo TB, Wan B, Zhou B, Zhang JZ. Association of CD4+CD25+Foxp3+ regulatory T cells with chronic activity and viral clearance in patients with hepatitis B. *Int Immunol* 2007; **19**: 133-140
- 60 **Stoop JN**, van der Molen RG, Kuipers EJ, Kusters JG, Janssen HL. Inhibition of viral replication reduces regulatory T cells and enhances the antiviral immune response in chronic hepatitis B. *Virology* 2007; **361**: 141-148
- 61 **Feng IC**, Koay LB, Sheu MJ, Kuo HT, Sun CS, Lee C, Chuang WL, Liao SK, Wang SL, Tang LY, Cheng CJ, Tsai SL. HBcAg-specific CD4+CD25+ regulatory T cells modulate immune tolerance and acute exacerbation on the natural history of chronic hepatitis B virus infection. *J Biomed Sci* 2007; **14**: 43-57
- 62 **Kondo Y**, Kobayashi K, Ueno Y, Shiina M, Niitsuma H, Kanno N, Kobayashi T, Shimosegawa T. Mechanism of T cell hyporesponsiveness to HBcAg is associated with regulatory T cells in chronic hepatitis B. *World J Gastroenterol* 2006; **12**: 4310-4317
- 63 **Toka FN**, Suvas S, Rouse BT. CD4+ CD25+ T cells regulate vaccine-generated primary and memory CD8+ T-cell responses against herpes simplex virus type 1. *J Virol* 2004; **78**: 13082-13089
- 64 **Furuichi Y**, Tokuyama H, Ueha S, Kurachi M, Moriyasu F, Kakimi K. Depletion of CD25+CD4+ T cells (Tregs) enhances the HBV-specific CD8+ T cell response primed by DNA immunization. *World J Gastroenterol* 2005; **11**: 3772-3777

S- Editor Ma N L- Editor Roberts SE E- Editor Ma WH

Robert Thimme, MD, Professor, Series Editor

Hepatitis C virus infection and apoptosis

Richard Fischer, Thomas Baumert, Hubert E Blum

Richard Fischer, Hubert E Blum, Department of Internal Medicine II, University of Freiburg, Germany

Thomas Baumert, Inserm U748, Service d'Hépatogastro-entérologie, Hôpitaux Universitaires de Strasbourg, Strasbourg, France

Correspondence to: Richard Fischer, MD, Department of Internal Medicine II, University of Freiburg, Hugstetter Strasse 55, D-79106 Freiburg, Germany. frisricha@medizin.uni-freiburg.de

Telephone: +49-761-2703403 Fax: +49-761-2703760

Received: June 26, 2007 Revised: July 9, 2007

Abstract

Apoptosis is central for the control and elimination of viral infections. In chronic hepatitis C virus (HCV) infection, enhanced hepatocyte apoptosis and upregulation of the death inducing ligands CD95/Fas, TRAIL and TNF α occur. Nevertheless, HCV infection persists in the majority of patients. The impact of apoptosis in chronic HCV infection is not well understood. It may be harmful by triggering liver fibrosis, or essential in interferon (IFN) induced HCV elimination. For virtually all HCV proteins, pro- and anti-apoptotic effects have been described, especially for the core and NS5A protein. To date, it is not known which HCV protein affects apoptosis *in vivo* and whether the infectious virions act pro- or anti-apoptotic. With the availability of an infectious tissue culture system, we now can address pathophysiologically relevant issues. This review focuses on the effect of HCV infection and different HCV proteins on apoptosis and of the corresponding signaling cascades.

© 2007 WJG. All rights reserved.

Key words: Hepatitis C; Spoptosis; TRAIL; CD95/Fas; TNF α ; Perforin

Fischer R, Baumert T, Blum HE. Hepatitis C virus infection and apoptosis. *World J Gastroenterol* 2007; 13(36): 4865-4872

<http://www.wjgnet.com/1007-9327/13/4865.asp>

INTRODUCTION

Hepatitis C virus (HCV) infection persists in approximately. Eighty percent of patients and is a leading cause of liver

cirrhosis and hepatocellular carcinoma^[1-4]. Worldwide, about 300 million individuals are HCV infected. The only antiviral treatment available to date with PEG-INF and ribavirin does not eliminate HCV infection in a large proportion of patients, especially in HCV genotype 1 infection, and, at the same time, has multiple severe side effects. With the availability of an infectious tissue culture system, we now can address pathophysiologically relevant issues for new treatment options^[1-3]. HCV belongs to the flaviviridae. It has an enveloped, positive strand RNA genome of 9.6 kb length containing one open reading frame translated into a single polypeptide. Posttranslational cleavage yields 4 structural (E1, E2, core, p7 (probably) and 6 nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, NS5B). Six different genotypes (1 [a, b, c], 2 [a, b, c], 3 [a, b], 4a, 5a, 6a) and 52 subtypes have been described. Due to the lack of proofreading function of the RNA-dependent RNA-polymerase (NS5B), HCV has a high mutation rate and exists as genetically heterogeneous quasispecies in individual patients^[5-7]. The different genotypes differ genetically from one another by at least 30%, and the different subtypes within a genotype by more than 20%. This genetic heterogeneity makes it difficult to compare apoptotic pathways obtained with different HCV genotypes. In general, apoptosis is central to viral clearance. In HCV-infected liver, however, despite enhanced hepatocyte apoptosis, viral persistence is observed.

APOPTOSIS IN HCV-INFECTED LIVER

Immune cell deficiency

The immune response to viral infections includes different components of the innate and the acquired immune system. They induce apoptosis as a host defense against viral infections. The innate immune system as the first line of defense directly activates inflammatory cells, such as macrophages (e.g., granulocytes, Kupffer cells in the liver) and natural killer (NK) cells which may directly cause death of the infected cells. On the other hand, viral RNA or proteins can bind to intracellular molecules that modulate or directly induce cell death^[8]. In this immune cell-independent, virus-induced apoptosis of the host cell protein kinase R (PKR)^[9,10] and the cytoplasmic RNA helicase RIG-I^[11] play central roles. RIG-I activates Cardif, a cytosolic protein that localizes to the mitochondrial membrane where it acts pro-apoptotic^[12,13]. PKR is also activated by interferons

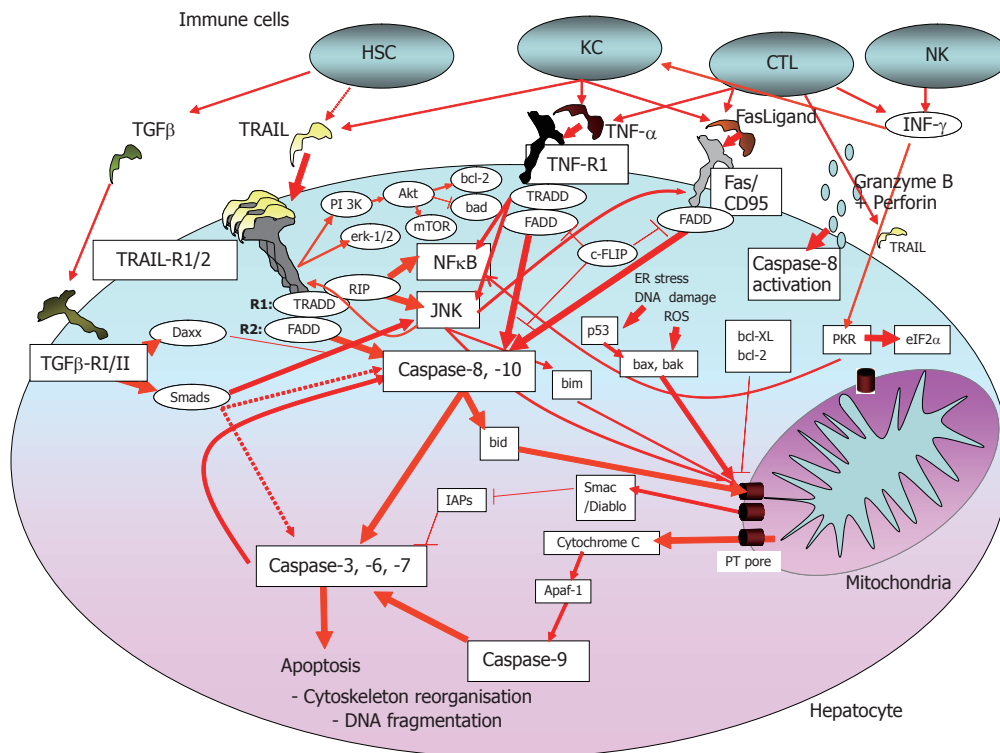


Figure 1 Signal transduction pathway of apoptosis in hepatocytes. Immune cells induce apoptosis in hepatocytes by death receptor ligands (TRAIL, TNF α , CD95Ligand, TGF- β) and granzyme B/perforin. Ligand-induced apoptosis activates caspase-8, whereas intrinsic apoptosis occurs via the mitochondrial permeability transition (PT) pore and activation of caspase-9. Caspase-9 and -8 activation converge in activation of the effector caspases-3, -6 and -7, resulting in irreversible apoptosis induction. HSC: Hepatic stellate cells; KC: Kupffer cells; CTL: Cytotoxic T-lymphocytes; NK: Natural killer cells.

(Figure 1) and acts *via* the downstream transcription factor eIF-2 α ^[14,15]. In HCV infection, the activated innate immune system alone is obviously insufficient to eliminate the virus^[16]. The acquired immune system consists of the humoral (antibody-secreting B-lymphocytes/plasma cells) and the cellular immune system (CD4 $^{+}$ - and CD8 $^{+}$ -T-lymphocytes). This system is essential for the clearance of most viral infections and depends on complex intercellular interactions and the recognition of viral antigens presented by specific cells (e.g., dendritic cells). CD4 $^{+}$ -T-lymphocytes activate CD8 $^{+}$ -T-lymphocytes, cytotoxic T lymphocytes (CTLs), macrophages and B-lymphocytes^[16]. The antigen-primed CD8 $^{+}$ -T-lymphocytes/CTLs directly kill infected cells *via* direct cell-cell-contact, and release of cytotoxic and/or antiviral cytokines (e.g., IFN γ , TNF α), whereas IFN γ and IFN α are also able to eliminate the virus without killing the host cell^[17,18]. In chronic HCV infection, the acquired immune system is, among others, impaired by T cell failure, dysfunction and exhaustion^[19]. This failure includes CD4 $^{+}$ - as well as CD8 $^{+}$ -T-lymphocytes.

ENHANCED HEPATOCYTE APOPTOSIS IN HCV INFECTION *IN VIVO*

Most of the cytotoxic effects mentioned above occur *via* programmed cell death, with activation of the intracellular suicide program through specific signals. Because chronic viral infection may reflect a failure of the immune system, specific apoptosis induction may not occur. In chronic HCV infection, however, enhanced hepatocyte apoptosis has been described, independent from the HCV genotype^[20]. Apoptosis varies between 0.54%^[20] and 20.00% of hepatocytes^[21], depending on the methods used. Typical pathomorphological features

of apoptosis (e.g., nuclear fragmentation, cell shrinkage) may be seen only in a minority of hepatocytes. The close physical proximity of apoptotic hepatocytes and infiltrating lymphocytes suggests an immune cell-mediated apoptosis^[20,22]. Apoptosis correlates with liver pathology^[20,21] and may contribute to fibrogenesis^[23]. Due to the difficulty to identify HCV infected hepatocytes, it is unknown whether apoptotic hepatocytes are indeed HCV infected. The number of HCV infected hepatocytes is in the range between 1% and 10%^[24]. Therefore, we actually do not know whether apoptosis is indeed related to HCV clearance. In an animal model of cholestasis, inhibition of hepatocyte apoptosis reduced fibrogenesis^[25] and excessive apoptosis lead to fulminant hepatitis^[26,27]. Therefore, anti-apoptotic therapy to prevent HCV-related liver damage has been suggested^[28,29]. By contrast, in a chimeric mouse-human model, pro-apoptotic gene therapy with proapoptotic Bid, engineered to contain a specific cleavage site for NS3/NS4A protease, results in a considerable decline of HCV RNA in serum^[30]. The relation between PEG-IFN/ribavirin-induced viral clearance and apoptosis of infected hepatocytes is largely unknown. INFs induce apoptosis in hepatoma cells, activate pro-apoptotic PKR^[10] and upregulate death receptor ligands. However, anti-apoptotic effects have also been described^[7,31-33].

LIGAND-INDUCED HEPATOCYTE APOPTOSIS IN HCV INFECTION

Hepatocytes most likely represent so-called type-II cells, for which external activation of the death signaling pathway often is insufficient to induce apoptosis. Here, apoptosis requires in addition amplification by the mitochondrial pathway (intrinsic apoptosis pathway). The

latter is affected by oxidative stress, DNA damage, and viral proteins (Figure 1).

Targeted apoptosis induction *via* CTLs and macrophages largely occurs *via* the ligands and receptors of the TNF α family: TNF α /TNF-receptor 1, CD95/CD95Ligand and TRAIL/Trail receptor-1 and -2, respectively (Figure 1). Ligand binding induces the formation of a death-inducing signaling complex, resulting in the activation of caspase-8 (caspases are the proteases involved in the apoptosis signaling cascade^[34]). Active caspase-8 can trigger two signaling pathways. The first pathway involves cleavage of bid, followed by mitochondria-dependent activation of caspase-9 *via* cytochrome C release and apaf-1^[35] (Figure 1). Mitochondria-dependent apoptosis is amplified by pro-apoptotic bax, bad, bak and others, while molecules like bcl-2 or bcl-XL act anti-apoptotic. These proteins converge at the mitochondrial permeability transition (PT) pore that regulates release of apoptotic regulatory proteins, e.g., procaspase-9, cytochrome C, apoptosis inducing factor (AIF) or Smac/Diablo^[36-38]. The second pathway involves caspase-8 activation that may bypass mitochondria resulting in the direct activation of effector caspases (caspase-3, -6, -7). Cellular inhibitors of apoptosis (IAPs, survivin, c-FLIP) are able to block caspase activation and apoptosis^[39] (Figure 1).

Growth-factor activated MAP-kinases Erk-1/2 and PKB/Akt inhibit apoptosis directly (e.g., through inactivation of pro-apoptotic bad) or *via* upregulation of anti-apoptotic proteins (e.g., bcl-2). By contrast, sustained stress activation of c-jun kinase (JNK) enhances death ligand-induced apoptosis *via* bim activation and consecutive mitochondrial apoptosis or *via* enhanced death-receptor membrane trafficking^[40-42]. Most death ligands, especially TNF α and TRAIL, activate NF κ B, which has anti-apoptotic effects in hepatocytes by upregulation of anti-apoptotic proteins, e.g., c-FLIP and bcl-XL^[43].

Death receptor ligands may be secreted by immune cells (e.g., macrophages) or may be membrane-bound. The latter form induces apoptosis more efficiently^[44]. In the normal liver, INF γ -activated Kupffer cells can kill neighbouring cells *via* TRAIL and CD95Ligand^[37,44]. By contrast, in injured liver, activated hepatic stellate cells release TGF- β that may induce apoptosis of hepatocytes^[45,46]. While TGF- β 1 expression is increased in the HCV-infected liver^[22], the impact of TGF- β on hepatocyte apoptosis in HCV-infected patients remains elusive. Apart from apoptosis induction, TGF- β is a key molecule in the pathogenesis of liver fibrosis^[47].

Hepatocytes undergo apoptosis in response to CD95Ligand and TNF α , whereas TRAIL presumably only induces apoptosis in infected or malignantly transformed hepatocytes/hepatoma cells, but not in normal liver cells. For all three death ligands, in chronic HCV infection, upregulation has been described^[20,48-51]. Further, HCV-specific CTL clones induced CD95Ligand-, TNF α - and perforin-dependent hepatocyte apoptosis^[52,53]. In HCV-infected liver, CD8+ T cells express CD95Ligand^[49] and TRAIL^[54] (Fischer, Blum Schmitt-Gräff *et al.*, unpublished data). Interestingly, CD95Ligand-induced apoptosis did not depend on HCV infection/antigen presentation, because bystander killing of non-HCV infected hepatocytes was

observed. TRAIL-induced apoptosis seems especially important in viral defense. Adenoviral-infected murine and human hepatocytes are sensitized to TRAIL-induced apoptosis, while CD95Ligand-induced cell death is not affected^[50,55]. In TRAIL knock-out mice resolution of pulmonary influenza infection is TRAIL-dependent^[56], and CMV infected colon epithelial cells or skin fibroblasts become sensitive to TRAIL-induced apoptosis^[57]. Further, in mice infected with encephalomyocarditis virus, blocking of TRAIL resulted in higher viral titers and early death^[58]. In concanamycin- and listeria-induced hepatitis, liver cell apoptosis is TRAIL-dependent^[59]. PEG-INF/ribavirin therapy of patients with chronic HCV infection results in a rapid and sustained TRAIL elevation, suggesting a role of TRAIL in viral clearance^[60]. Similar observations have been made for soluble CD95Ligand^[61,33]. Therefore, TRAIL-induced apoptosis may play a major role in HCV defense and elimination.

Another mechanism of apoptosis involves the release of granzyme B and perforin by CTLs^[62,63]. Exocytosed perforins form transmembrane channels in the target cell that allow the entry of granzyme B. Similar to death-ligand induced apoptosis, granzyme B-mediated apoptosis largely depends on caspase activation and the sensitivity of the target cell. Hepatocytes seem resistant to granzyme B mediated cell death, and CTL killing of infected hepatocytes is perforin/granzyme B- independent^[29,64]. Therefore, a contribution of this apoptosis mechanism in patients with viral hepatitis is very unlikely.

MODIFIED HEPATOCYTE APOPTOSIS *IN VITRO*

Viral proteins interfere with the cellular apoptotic signaling pathway and block key cellular elements of the host cell. Until recently, the lack of an infectious HCV tissue culture system did not allow to study the impact of HCV infection on hepatocyte apoptosis. Overall, the data regarding the role of different HCV proteins are controversial and ascribe to a given viral protein pro- and anti-apoptotic effects, depending on the experimental system used. Since in most models viral proteins are overexpressed by non-viral promoters, for virtually all HCV proteins a pro-apoptotic effect has been described. Apart from the unphysiological expression of viral proteins, these models further lack the balance of intracellular viral expression of the different HCV proteins and their interactions. Especially in HCV infection, intracellular viral protein expression is very low.

Further, HCV is genetically highly variable and exists as quasispecies in a given patient. Different pro- and anti-apoptotic effects of the HCV core protein from an individual patient have been described^[65], suggesting special properties of different quasispecies proteins. These protein differences may explain in part the different effects of viral proteins on apoptosis. Studies of the contribution of genotypes or quasispecies to the effects on apoptosis are largely missing. Further, experiments designed to study the impact of HCV infection on hepatocyte apoptosis must also consider the interactions between the different

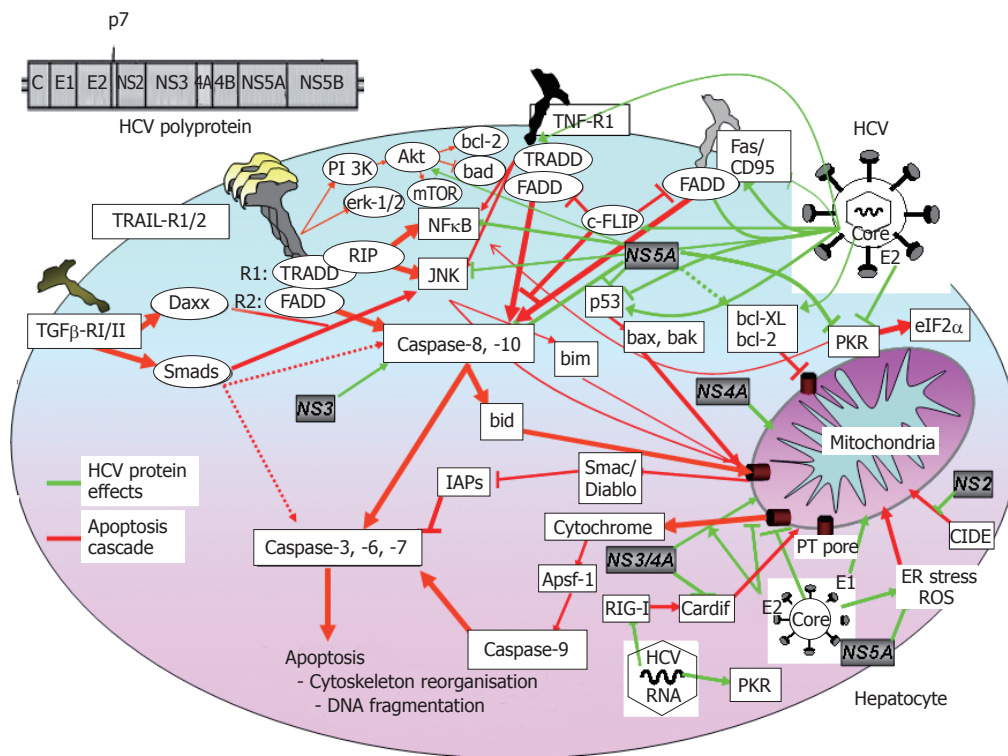


Figure 2 Interference of HCV proteins with the apoptosis cascade. Pro- and anti-apoptotic effects of HCV proteins converge at the mitochondria (e.g., NS2, NS3/4A, NS5A, E2, core), partly indirectly via p53 (NS5A, core) and activation of PKB/Akt, c-Jun kinase JNK (core) or NF κ B (NS5A). HCV interacts directly with death receptors (core), the corresponding death receptor domains (FADD) and caspase-8 (NS5A). HCV double-strand RNA-activated protein-kinase R (PKR) induced signaling via RIG-I (retinoic acid inducible gene-I) and Cardif is directly (E2, NS5A) and indirectly (NS3/4A) disturbed.

HCV proteins. Therefore, only models based on the complete and infectious virus may reflect to some extent the *in vivo* situation.

HCV core protein

The structural HCV core protein makes up the virion nucleocapsid^[1,5,66]. The core protein has been shown to affect various cellular signaling pathways^[67] and to activate different promoters, e.g., c-myc, c-fos^[68-70]. It has further been shown to have pro- and anti-apoptotic effects in death ligand-mediated hepatocyte apoptosis. Core-dependent inhibition of TNF- α ^[71] and CD95Ligand-induced apoptosis^[72] has been described in a hepatoma cell line. In other models, overexpressed HCV core protein did not prevent CD95Ligand-induced apoptosis in hepatoma cells^[73] or transgenic mice expressing HCV core protein, E1, E2 and NS2, respectively. HCV core protein inhibits CD95Ligand-mediated apoptosis by prevention of cytochrome C release from mitochondria and consecutive activation of caspase-9, -3 and -7^[74]. Direct physical and pro-apoptotic interaction of the core protein with the cytoplasmatic domains of CD95, TNF-R1^[75] and lymphotoxin- β ^[76] receptors have been reported. Further, direct binding to the downstream death domain of FADD and c-FLIP^[77] has been shown to result in anti-apoptotic effects. Recently, inhibition of the TGF- β -pathway by direct interaction of the core protein with the DNA-binding domain of Smad3, important apoptosis mediators of TGF- β -receptor-I/II, has been demonstrated^[65].

Several studies demonstrated binding of the HCV core protein to p53, either inhibiting or activating p53^[69,78-80] with consecutive anti- or pro-apoptotic effects. In some studies apoptosis was inhibited in hepatoma through core-dependent phosphorylation and activation of STAT3 that induces the anti-apoptotic bcl-XL^[81,82]. Other studies

showed core-induced apoptosis through mitochondrial cytochrome C release and indirect activation of bax^[83,84]. TRAIL-induced apoptosis in hepatoma cells seems enhanced by core-dependent bid-cleavage^[83]. Mitochondrial functions are altered by core-induced oxidative stress, making cells more susceptible to apoptosis^[85]. Machida *et al*^[86] showed HCV-dependent production of reactive oxygen species (ROS), lowering of the mitochondrial transmembrane potential and consecutive caspase-independent cell death.

Taken together, it remains unclear whether HCV core protein inhibits or induces death receptor-mediated apoptosis of hepatocytes (Figure 2).

HCV envelope proteins E1 and E2

HCV proteins E1 and E2 are envelope proteins, that mediate viral binding and entry^[7,87]. In a transgenic mouse model expressing HCV proteins, CD95Ligand-mediated hepatocyte apoptosis is inhibited by E1, E2, NS2 and core, respectively. The activation of mitochondrial apoptosis (intrinsic pathway) is involved, because release of cytochrome C and caspase-9, but not caspase-8 activation are inhibited. To date, the contribution of the individual HCV proteins was not investigated^[74]. In E1-expressing hepatoma cells, apoptosis depends on the presence of the C-terminal transmembrane domain of E1, presumably altering membrane permeability of E1^[88,89].

Inhibition of TRAIL-induced apoptosis in hepatoma cells by E2, presumably through inhibition of mitochondrial cytochrome C release has been demonstrated^[90], while E1 had no effect and core did not counteract the anti-apoptotic effect of E2. Comparable results were obtained in core-E1-E2 transfected hepatoma cells or transgenic mice. In both models, core-E1-E2 induced less apoptosis than core-transfected

cells/transgenic mice and controls, respectively^[91]. By contrast, E2 induces mitochondria-related and caspase-dependent apoptosis in the same hepatoma cell line^[92]. These controversial data may reflect the use of different promoters that overexpress E2, while at the same time, the HCV genotype or the individual sequence of E2 have not been considered. Therefore, it still remains unclear whether HCV E1 has apoptosis-modulating activity *in vivo*, and whether HCV E2 acts anti- or pro-apoptotic (Figure 2).

HCV nonstructural proteins

The non-structural HCV proteins NS2 and NS3 are the two viral proteases required for posttranslational cleavage of non-structural proteins. NS2 is a transmembrane protein localized in the endoplasmic reticulum (ER) that directly binds and inhibits CIDE-B-induced apoptosis (cell death-inducing DFF45 (DNA-fragmentation-factor)-like effector^[93]). CIDE-B-induced apoptosis is assumed to occur *via* the mitochondrial pathway^[94,95]. Its role in hepatocyte apoptosis and viral hepatitis remain to be determined, however.

NS3 has a helicase and NTPase activity that are involved in RNA replication^[7]. Importantly, NS3 prevents viral RNA-induced pro-apoptotic RIG-I effects by specific cleavage of downstream Cardif, a protein that translocates to the mitochondrial membrane when activated^[13]. The precise role of Cardif in hepatocyte apoptosis and viral hepatitis is unknown, however. In contrast, NS3 induces caspase-8 dependent apoptosis in hepatocytes^[96] and in dendritic cells^[97]; the underlying mechanism remains unknown.

HCV NS4A is a cofactor that binds to NS3. NS4A alone and complexed with NS3 is localized in mitochondria and induces the release of cytochrome C and caspase-8 independent apoptosis^[98]. NS4B is an integral ER membrane protein that may play a role in anchoring the replication complex^[6,7]. A role in the apoptotic signaling pathway has not yet been described.

The function of NS5A is not yet well defined. NS5A interferes with the response to IFN and seems to play an important role in viral replication^[5,7]. NS5A has sequence homologies with bcl-2 and binds to FKBP38, thereby augmenting the anti-apoptotic effect of bcl-2^[99] and inhibiting the pro-apoptotic action of bax in hepatoma cells^[100]. Anti-apoptotic effects of NS5A are further mediated by cytoplasmatic sequestering of p53^[101], activation of PI3-kinase-Akt/PKB survival pathway^[102], activation of STAT3 with enhanced expression of bcl-XL and p21^[103] and activation of NFκB^[104]. By contrast, the direct inhibition of pro-apoptotic bin1, a tumor suppressor protein with a SH3 domain, has been described in hepatoma cells^[105], and a direct NS5A-induced apoptosis has also been shown^[97,106]. NS5B is the viral RNA-dependent RNA polymerase^[5-7]. There are no studies demonstrating a role of NS5B in apoptosis of hepatocytes/hepatoma cells, while a pro-apoptotic effect of NS5B has been demonstrated in dendritic cells^[97].

In conclusion, similar to HCV structural proteins, the effect of non-structural proteins on hepatocyte apoptosis *in vivo* remains unclear.

CONCLUSION

The role of apoptosis in HCV infection is not well defined. Kinetics and extent of hepatocyte apoptosis as well as the pro- and anti-apoptotic mechanisms involved remain unclear. It remains further unclear whether enhanced hepatocyte apoptosis in HCV infection is related to viral clearance, and whether it has a therapeutic benefit.

Most experimental models have fundamental shortcomings and there are no data from primary hepatocytes, tissue cultures or animal models. The majority of the data were obtained with different tumor cell lines that may in themselves be inhomogeneous. Different HCV genotypes and quasispecies may induce different effects, and most studies employ nonphysiologically overexpressed viral proteins. In HCV infected patients, by comparison, only very low quantities of HCV proteins are detectable, and the balanced expression of these proteins may be essential. Therefore, the results obtained to date have to be interpreted with great caution. The now available infectious tissue culture systems^[1-3] as well as future *in vivo* model systems may give answers to these questions, may better reflect the *in vivo* situation and may help to clarify the interference of HCV with apoptotic pathways and its role in the pathogenesis of HCV infection and clearance.

REFERENCES

- 1 **Wakita T**, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, Murthy K, Habermann A, Kräusslich HG, Mizokami M, Bartenschlager R, Liang TJ. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* 2005; **11**: 791-796
- 2 **Lohmann V**, Hoffmann S, Herian U, Penin F, Bartenschlager R. Viral and cellular determinants of hepatitis C virus RNA replication in cell culture. *J Virol* 2003; **77**: 3007-3019
- 3 **Lindenbach BD**, Evans MJ, Syder AJ, Wölk B, Tellinghuisen TL, Liu CC, Maruyama T, Hynes RO, Burton DR, McKeating JA, Rice CM. Complete replication of hepatitis C virus in cell culture. *Science* 2005; **309**: 623-626
- 4 **Lauer GM**, Walker BD. Hepatitis C virus infection. *N Engl J Med* 2001; **345**: 41-52
- 5 **Bartenschlager R**. Hepatitis C virus molecular clones: from cDNA to infectious virus particles in cell culture. *Curr Opin Microbiol* 2006; **9**: 416-422
- 6 **Dustin LB**, Rice CM. Flying under the radar: the immunobiology of hepatitis C. *Annu Rev Immunol* 2007; **25**: 71-99
- 7 **Pavio N**, Lai MM. The hepatitis C virus persistence: how to evade the immune system? *J Biosci* 2003; **28**: 287-304
- 8 **Balachandran S**, Roberts PC, Kipperman T, Bhalla KN, Compans RW, Archer DR, Barber GN. Alpha/beta interferons potentiate virus-induced apoptosis through activation of the FADD/Caspase-8 death signaling pathway. *J Virol* 2000; **74**: 1513-1523
- 9 **Zhang P**, Samuel CE. Protein kinase PKR plays a stimulus- and virus-dependent role in apoptotic death and virus multiplication in human cells. *J Virol* 2007; **81**: 8192-8200
- 10 **García MA**, Gil J, Ventoso I, Guerra S, Domingo E, Rivas C, Esteban M. Impact of protein kinase PKR in cell biology: from antiviral to antiproliferative action. *Microbiol Mol Biol Rev* 2006; **70**: 1032-1060
- 11 **Yoneyama M**, Fujita T. Function of RIG-I-like receptors in antiviral innate immunity. *J Biol Chem* 2007; **282**: 15315-15318
- 12 **Herzer K**, Sprinzl MF, Galle PR. Hepatitis viruses: live and let die. *Liver Int* 2007; **27**: 293-301
- 13 **Meylan E**, Curran J, Hofmann K, Moradpour D, Binder M,

- Bartenschlager R, Tschopp J. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature* 2005; **437**: 1167-1172
- 14 **Der SD**, Yang YL, Weissmann C, Williams BR. A double-stranded RNA-activated protein kinase-dependent pathway mediating stress-induced apoptosis. *Proc Natl Acad Sci USA* 1997; **94**: 3279-3283
 - 15 **Gil J**, Esteban M. Induction of apoptosis by the dsRNA-dependent protein kinase (PKR): mechanism of action. *Apoptosis* 2000; **5**: 107-114
 - 16 **Su AI**, Pezacki JP, Wodicka L, Brideau AD, Supekova L, Timme R, Wieland S, Bukh J, Purcell RH, Schultz PG, Chisari FV. Genomic analysis of the host response to hepatitis C virus infection. *Proc Natl Acad Sci USA* 2002; **99**: 15669-15674
 - 17 **Frese M**, Schwärzle V, Barth K, Krieger N, Lohmann V, Mihm S, Haller O, Bartenschlager R. Interferon-gamma inhibits replication of subgenomic and genomic hepatitis C virus RNAs. *Hepatology* 2002; **35**: 694-703
 - 18 **Guidotti LG**, Chisari FV. Noncytolytic control of viral infections by the innate and adaptive immune response. *Annu Rev Immunol* 2001; **19**: 65-91
 - 19 **Timme R**, Bukh J, Spangenberg HC, Wieland S, Pemberton J, Steiger C, Govindarajan S, Purcell RH, Chisari FV. Viral and immunological determinants of hepatitis C virus clearance, persistence, and disease. *Proc Natl Acad Sci USA* 2002; **99**: 15661-15668
 - 20 **Calabrese F**, Pontisso P, Pettenazzo E, Benvegnù L, Vario A, Chemello L, Alberti A, Valente M. Liver cell apoptosis in chronic hepatitis C correlates with histological but not biochemical activity or serum HCV-RNA levels. *Hepatology* 2000; **31**: 1153-1159
 - 21 **Bantel H**, Lügering A, Poremba C, Lügering N, Held J, Domschke W, Schulze-Osthoff K. Caspase activation correlates with the degree of inflammatory liver injury in chronic hepatitis C virus infection. *Hepatology* 2001; **34**: 758-767
 - 22 **Lau JY**, Xie X, Lai MM, Wu PC. Apoptosis and viral hepatitis. *Semin Liver Dis* 1998; **18**: 169-176
 - 23 **Canbay A**, Friedman S, Gores GJ. Apoptosis: the nexus of liver injury and fibrosis. *Hepatology* 2004; **39**: 273-278
 - 24 **Hiramatsu N**, Hayashi N, Haruna Y, Kasahara A, Fusamoto H, Mori C, Fuke I, Okayama H, Kamada T. Immunohistochemical detection of hepatitis C virus-infected hepatocytes in chronic liver disease with monoclonal antibodies to core, envelope and NS3 regions of the hepatitis C virus genome. *Hepatology* 1992; **16**: 306-311
 - 25 **Canbay A**, Feldstein A, Baskin-Bey E, Bronk SF, Gores GJ. The caspase inhibitor IDN-6556 attenuates hepatic injury and fibrosis in the bile duct ligated mouse. *J Pharmacol Exp Ther* 2004; **308**: 1191-1196
 - 26 **Ogasawara J**, Watanabe-Fukunaga R, Adachi M, Matsuzawa A, Kasugai T, Kitamura Y, Itoh N, Suda T, Nagata S. Lethal effect of the anti-Fas antibody in mice. *Nature* 1993; **364**: 806-809
 - 27 **Kohli V**, Selzner M, Madden JF, Bentley RC, Clavien PA. Endothelial cell and hepatocyte deaths occur by apoptosis after ischemia-reperfusion injury in the rat liver. *Transplantation* 1999; **67**: 1099-1105
 - 28 **Feldstein AE**, Gores GJ. An apoptosis biomarker goes to the HCV clinic. *Hepatology* 2004; **40**: 1044-1046
 - 29 **Guicciardi ME**, Gores GJ. Apoptosis: a mechanism of acute and chronic liver injury. *Gut* 2005; **54**: 1024-1033
 - 30 **Hsu EC**, Hsi B, Hirota-Tsuchihara M, Ruland J, Iorio C, Sarangi F, Diao J, Migliaccio G, Tyrrell DL, Kneteman N, Richardson CD. Modified apoptotic molecule (BID) reduces hepatitis C virus infection in mice with chimeric human livers. *Nat Biotechnol* 2003; **21**: 519-525
 - 31 **Maher SG**, Romero-Weaver AL, Scarzello AJ, Gamero AM. Interferon: cellular executioner or white knight? *Curr Med Chem* 2007; **14**: 1279-1289
 - 32 **Yano H**, Ogasawara S, Momosaki S, Akiba J, Kojiro S, Fukahori S, Ishizaki H, Kuratomi K, Basaki Y, Oie S, Kuwano M, Kojiro M. Growth inhibitory effects of pegylated IFN alpha-2b on human liver cancer cells *in vitro* and *in vivo*. *Liver Int* 2006; **26**: 964-975
 - 33 **Yoneyama K**, Goto T, Miura K, Mikami K, Ohshima S, Nakane K, Lin JG, Sugawara M, Nakamura N, Shirakawa K, Komatsu M, Watanabe S. The expression of Fas and Fas ligand, and the effects of interferon in chronic liver diseases with hepatitis C virus. *Hepatol Res* 2002; **24**: 327-337
 - 34 **Kumar S**. Caspase function in programmed cell death. *Cell Death Differ* 2007; **14**: 32-43
 - 35 **Schaefer U**, Voloshanenko O, Willen D, Walczak H. TRAIL: a multifunctional cytokine. *Front Biosci* 2007; **12**: 3813-3824
 - 36 **Eichhorst ST**. Modulation of apoptosis as a target for liver disease. *Expert Opin Ther Targets* 2005; **9**: 83-99
 - 37 **Fischer R**, Schmitt M, Bode JG, Häussinger D. Expression of the peripheral-type benzodiazepine receptor and apoptosis induction in hepatic stellate cells. *Gastroenterology* 2001; **120**: 1212-1226
 - 38 **Brenner C**, Grimm S. The permeability transition pore complex in cancer cell death. *Oncogene* 2006; **25**: 4744-4756
 - 39 **Deveraux QL**, Reed JC. IAP family proteins--suppressors of apoptosis. *Genes Dev* 1999; **13**: 239-252
 - 40 **Corazza N**, Jakob S, Schaefer C, Frese S, Keogh A, Stroka D, Kassahn D, Torgler R, Mueller C, Schneider P, Brunner T. TRAIL receptor-mediated JNK activation and Bim phosphorylation critically regulate Fas-mediated liver damage and lethality. *J Clin Invest* 2006; **116**: 2493-2499
 - 41 **Graf D**, Kurz AK, Fischer R, Reinehr R, Häussinger D. Taurolithocholic acid-3 sulfate induces CD95 trafficking and apoptosis in a c-Jun N-terminal kinase-dependent manner. *Gastroenterology* 2002; **122**: 1411-1427
 - 42 **Wada T**, Penninger JM. Mitogen-activated protein kinases in apoptosis regulation. *Oncogene* 2004; **23**: 2838-2849
 - 43 **Wullaert A**, Heynink K, Beyaert R. Mechanisms of crosstalk between TNF-induced NF-kappaB and JNK activation in hepatocytes. *Biochem Pharmacol* 2006; **72**: 1090-1101
 - 44 **Fischer R**, Carriers A, Reinehr R, Häussinger D. Caspase 9-dependent killing of hepatic stellate cells by activated Kupffer cells. *Gastroenterology* 2002; **123**: 845-861
 - 45 **Lee KY**, Bae SC. TGF-beta-dependent cell growth arrest and apoptosis. *J Biochem Mol Biol* 2002; **35**: 47-53
 - 46 **Oberhammer FA**, Pavelka M, Sharma S, Tiefenbacher R, Purchio AF, Bursch W, Schulte-Hermann R. Induction of apoptosis in cultured hepatocytes and in regressing liver by transforming growth factor beta 1. *Proc Natl Acad Sci USA* 1992; **89**: 5408-5412
 - 47 **Friedman SL**. Molecular regulation of hepatic fibrosis, an integrated cellular response to tissue injury. *J Biol Chem* 2000; **275**: 2247-2250
 - 48 **Ghavami S**, Hashemi M, Kadhoda K, Alavian SM, Bay GH, Los M. Apoptosis in liver diseases--detection and therapeutic applications. *Med Sci Monit* 2005; **11**: RA337-RA345
 - 49 **Mita E**, Hayashi N, Iio S, Takehara T, Hijioka T, Kasahara A, Fusamoto H, Kamada T. Role of Fas ligand in apoptosis induced by hepatitis C virus infection. *Biochem Biophys Res Commun* 1994; **204**: 468-474
 - 50 **Mundt B**, Kühnel F, Zender L, Paul Y, Tillmann H, Trautwein C, Manns MP, Kubicka S. Involvement of TRAIL and its receptors in viral hepatitis. *FASEB J* 2003; **17**: 94-96
 - 51 **Zylberberg H**, Rimaniol AC, Pol S, Masson A, De Groote D, Berthelot P, Bach JF, Bréchet C, Zavala F. Soluble tumor necrosis factor receptors in chronic hepatitis C: a correlation with histological fibrosis and activity. *J Hepatol* 1999; **30**: 185-191
 - 52 **Ando K**, Hiroishi K, Kaneko T, Moriyama T, Muto Y, Kayagaki N, Yagita H, Okumura K, Imawari M. Perforin, Fas/Fas ligand, and TNF-alpha pathways as specific and bystander killing mechanisms of hepatitis C virus-specific human CTL. *J Immunol* 1997; **158**: 5283-5291
 - 53 **Gremion C**, Grabscheid B, Wölk B, Moradpour D, Reichen J, Pichler W, Cerny A. Cytotoxic T lymphocytes derived from patients with chronic hepatitis C virus infection kill bystander cells via Fas-FasL interaction. *J Virol* 2004; **78**: 2152-2157
 - 54 **Saitou Y**, Shiraki K, Fuke H, Inoue T, Miyashita K, Yamanaka Y, Yamaguchi Y, Yamamoto N, Ito K, Sugimoto K, Nakano T. Involvement of tumor necrosis factor-related apoptosis-

- inducing ligand and tumor necrosis factor-related apoptosis-inducing ligand receptors in viral hepatic diseases. *Hum Pathol* 2005; **36**: 1066-1073
- 55 **Mundt B**, Wirth T, Zender L, Waltemathe M, Trautwein C, Manns MP, Kühnel F, Kubicka S. Tumour necrosis factor related apoptosis inducing ligand (TRAIL) induces hepatic steatosis in viral hepatitis and after alcohol intake. *Gut* 2005; **54**: 1590-1596
 - 56 **Ishikawa E**, Nakazawa M, Yoshinari M, Minami M. Role of tumor necrosis factor-related apoptosis-inducing ligand in immune response to influenza virus infection in mice. *J Virol* 2005; **79**: 7658-7663
 - 57 **Sträter J**, Walczak H, Pukrop T, Von Müller L, Hasel C, Kornmann M, Mertens T, Möller P. TRAIL and its receptors in the colonic epithelium: a putative role in the defense of viral infections. *Gastroenterology* 2002; **122**: 659-666
 - 58 **Sedger LM**, Shows DM, Blanton RA, Peschon JJ, Goodwin RG, Cosman D, Wiley SR. IFN-gamma mediates a novel antiviral activity through dynamic modulation of TRAIL and TRAIL receptor expression. *J Immunol* 1999; **163**: 920-926
 - 59 **Zheng SJ**, Wang P, Tsabary G, Chen YH. Critical roles of TRAIL in hepatic cell death and hepatic inflammation. *J Clin Invest* 2004; **113**: 58-64
 - 60 **Pelli N**, Torre F, Delfino A, Basso M, Picciotto A. Soluble tumor necrosis factor-related ligand (sTRAIL) levels and kinetics during antiviral treatment in chronic hepatitis C. *J Interferon Cytokine Res* 2006; **26**: 119-123
 - 61 **Toyoda M**, Kakizaki S, Horiguchi N, Sato K, Takayama H, Takagi H, Nagamine T, Mori M. Role of serum soluble Fas/ soluble Fas ligand and TNF-alpha on response to interferon-alpha therapy in chronic hepatitis C. *Liver* 2000; **20**: 305-311
 - 62 **Lowin B**, Hahne M, Mattmann C, Tschopp J. Cytolytic T-cell cytotoxicity is mediated through perforin and Fas lytic pathways. *Nature* 1994; **370**: 650-652
 - 63 **Kägi D**, Vignaux F, Ledermann B, Bürki K, Depraetere V, Nagata S, Hengartner H, Golstein P. Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. *Science* 1994; **265**: 528-530
 - 64 **Kafrouni MI**, Brown GR, Thiele DL. Virally infected hepatocytes are resistant to perforin-dependent CTL effector mechanisms. *J Immunol* 2001; **167**: 1566-1574
 - 65 **Pavio N**, Battaglia S, Boucreux D, Arnulf B, Sobesky R, Hermine O, Brechot C. Hepatitis C virus core variants isolated from liver tumor but not from adjacent non-tumor tissue interact with Smad3 and inhibit the TGF-beta pathway. *Oncogene* 2005; **24**: 6119-6132
 - 66 **Baumert TF**, Ito S, Wong DT, Liang TJ. Hepatitis C virus structural proteins assemble into viruslike particles in insect cells. *J Virol* 1998; **72**: 3827-3836
 - 67 **Lai MM**, Ware CF. Hepatitis C virus core protein: possible roles in viral pathogenesis. *Curr Top Microbiol Immunol* 2000; **242**: 117-134
 - 68 **Ray RB**, Lagging LM, Meyer K, Steele R, Ray R. Transcriptional regulation of cellular and viral promoters by the hepatitis C virus core protein. *Virus Res* 1995; **37**: 209-220
 - 69 **Ray RB**, Steele R, Meyer K, Ray R. Transcriptional repression of p53 promoter by hepatitis C virus core protein. *J Biol Chem* 1997; **272**: 10983-10986
 - 70 **Ray RB**, Steele R, Meyer K, Ray R. Hepatitis C virus core protein represses p21WAF1/Cip1/Sid1 promoter activity. *Gene* 1998; **208**: 331-336
 - 71 **Ray RB**, Meyer K, Steele R, Shrivastava A, Aggarwal BB, Ray R. Inhibition of tumor necrosis factor (TNF-alpha)-mediated apoptosis by hepatitis C virus core protein. *J Biol Chem* 1998; **273**: 2256-2259
 - 72 **Ruggieri A**, Harada T, Matsuura Y, Miyamura T. Sensitization to Fas-mediated apoptosis by hepatitis C virus core protein. *Virology* 1997; **229**: 68-76
 - 73 **Dumoulin FL**, vsn dem Bussche A, Söhne J, Sauerbruch T, Spengler U. Hepatitis C virus core protein does not inhibit apoptosis in human hepatoma cells. *Eur J Clin Invest* 1999; **29**: 940-946
 - 74 **Machida K**, Tsukiyama-Kohara K, Seike E, Toné S, Shibasaki F, Shimizu M, Takahashi H, Hayashi Y, Funata N, Taya C, Yonekawa H, Kohara M. Inhibition of cytochrome c release in Fas-mediated signaling pathway in transgenic mice induced to express hepatitis C viral proteins. *J Biol Chem* 2001; **276**: 12140-12146
 - 75 **Zhu N**, Khoshnan A, Schneider R, Matsumoto M, Dennert G, Ware C, Lai MM. Hepatitis C virus core protein binds to the cytoplasmic domain of tumor necrosis factor (TNF) receptor 1 and enhances TNF-induced apoptosis. *J Virol* 1998; **72**: 3691-3697
 - 76 **Matsumoto M**, Hsieh TY, Zhu N, VanArsdale T, Hwang SB, Jeng KS, Gorbelenya AE, Lo SY, Ou JH, Ware CF, Lai MM. Hepatitis C virus core protein interacts with the cytoplasmic tail of lymphotoxin-beta receptor. *J Virol* 1997; **71**: 1301-1309
 - 77 **Saito K**, Meyer K, Warner R, Basu A, Ray RB, Ray R. Hepatitis C virus core protein inhibits tumor necrosis factor alpha-mediated apoptosis by a protective effect involving cellular FLICE inhibitory protein. *J Virol* 2006; **80**: 4372-4379
 - 78 **Herzer K**, Weyer S, Krammer PH, Galle PR, Hofmann TG. Hepatitis C virus core protein inhibits tumor suppressor protein promyelocytic leukemia function in human hepatoma cells. *Cancer Res* 2005; **65**: 10830-10837
 - 79 **Kao CF**, Chen SY, Chen JY, Wu Lee YH. Modulation of p53 transcription regulatory activity and post-translational modification by hepatitis C virus core protein. *Oncogene* 2004; **23**: 2472-2483
 - 80 **Otsuka M**, Kato N, Lan K, Yoshida H, Kato J, Goto T, Shiratori Y, Omata M. Hepatitis C virus core protein enhances p53 function through augmentation of DNA binding affinity and transcriptional ability. *J Biol Chem* 2000; **275**: 34122-34130
 - 81 **Otsuka M**, Kato N, Taniguchi H, Yoshida H, Goto T, Shiratori Y, Omata M. Hepatitis C virus core protein inhibits apoptosis via enhanced Bcl-xL expression. *Virology* 2002; **296**: 84-93
 - 82 **Yoshida T**, Hanada T, Tokuhisa T, Kosai K, Sata M, Kohara M, Yoshimura A. Activation of STAT3 by the hepatitis C virus core protein leads to cellular transformation. *J Exp Med* 2002; **196**: 641-653
 - 83 **Chou AH**, Tsai HF, Wu YY, Hu CY, Hwang LH, Hsu PI, Hsu PN. Hepatitis C virus core protein modulates TRAIL-mediated apoptosis by enhancing Bid cleavage and activation of mitochondria apoptosis signaling pathway. *J Immunol* 2005; **174**: 2160-2166
 - 84 **Lee SH**, Kim YK, Kim CS, Seol SK, Kim J, Cho S, Song YL, Bartschlag R, Jang SK. E2 of hepatitis C virus inhibits apoptosis. *J Immunol* 2005; **175**: 8226-8235
 - 85 **Okuda M**, Li K, Beard MR, Showalter LA, Scholle F, Lemon SM, Weinman SA. Mitochondrial injury, oxidative stress, and antioxidant gene expression are induced by hepatitis C virus core protein. *Gastroenterology* 2002; **122**: 366-375
 - 86 **Machida K**, Cheng KT, Lai CK, Jeng KS, Sung VM, Lai MM. Hepatitis C virus triggers mitochondrial permeability transition with production of reactive oxygen species, leading to DNA damage and STAT3 activation. *J Virol* 2006; **80**: 7199-7207
 - 87 **Barth H**, Liang TJ, Baumert TF. Hepatitis C virus entry: molecular biology and clinical implications. *Hepatology* 2006; **44**: 527-535
 - 88 **Ciccaglione AR**, Marcantonio C, Costantino A, Equestre M, Rapicetta M. Expression of HCV E1 protein in baculovirus-infected cells: effects on cell viability and apoptosis induction. *Intervirology* 2003; **46**: 121-126
 - 89 **Ciccaglione AR**, Marcantonio C, Tritarelli E, Equestre M, Magurano F, Costantino A, Nicoletti L, Rapicetta M. The transmembrane domain of hepatitis C virus E1 glycoprotein induces cell death. *Virus Res* 2004; **104**: 1-9
 - 90 **Lee SK**, Park SO, Joe CO, Kim YS. Interaction of HCV core protein with 14-3-3 protein releases Bax to activate apoptosis. *Biochem Biophys Res Commun* 2007; **352**: 756-762
 - 91 **Kamegaya Y**, Hiasa Y, Zukerberg L, Fowler N, Blackard JT, Lin W, Choe WH, Schmidt EV, Chung RT. Hepatitis C virus acts as a tumor accelerator by blocking apoptosis in a mouse model of hepatocarcinogenesis. *Hepatology* 2005; **41**: 660-667
 - 92 **Chiou HL**, Hsieh YS, Hsieh MR, Chen TY. HCV E2 may

- induce apoptosis of Huh-7 cells via a mitochondrial-related caspase pathway. *Biochem Biophys Res Commun* 2006; **345**: 453-458
- 93 **Viswakarma N**, Yu S, Naik S, Kashireddy P, Matsumoto K, Sarkar J, Surapureddi S, Jia Y, Rao MS, Reddy JK. Transcriptional regulation of Cidea, mitochondrial cell death-inducing DNA fragmentation factor alpha-like effector A, in mouse liver by peroxisome proliferator-activated receptor alpha and gamma. *J Biol Chem* 2007; **282**: 18613-18624
 - 94 **Inohara N**, Koseki T, Chen S, Wu X, Núñez G. CIDE, a novel family of cell death activators with homology to the 45 kDa subunit of the DNA fragmentation factor. *EMBO J* 1998; **17**: 2526-2533
 - 95 **Erdtmann L**, Franck N, Lerat H, Le Seyec J, Gilot D, Cannie I, Gripon P, Hibner U, Guguen-Guillouzo C. The hepatitis C virus NS2 protein is an inhibitor of CIDE-B-induced apoptosis. *J Biol Chem* 2003; **278**: 18256-18264
 - 96 **Prikhod'ko EA**, Prikhod'ko GG, Siegel RM, Thompson P, Major ME, Cohen JI. The NS3 protein of hepatitis C virus induces caspase-8-mediated apoptosis independent of its protease or helicase activities. *Virology* 2004; **329**: 53-67
 - 97 **Siavoshian S**, Abraham JD, Thumann C, Kieny MP, Schuster C. Hepatitis C virus core, NS3, NS5A, NS5B proteins induce apoptosis in mature dendritic cells. *J Med Virol* 2005; **75**: 402-411
 - 98 **Nomura-Takigawa Y**, Nagano-Fujii M, Deng L, Kitazawa S, Ishido S, Sada K, Hotta H. Non-structural protein 4A of Hepatitis C virus accumulates on mitochondria and renders the cells prone to undergoing mitochondria-mediated apoptosis. *J Gen Virol* 2006; **87**: 1935-1945
 - 99 **Wang J**, Tong W, Zhang X, Chen L, Yi Z, Pan T, Hu Y, Xiang L, Yuan Z. Hepatitis C virus non-structural protein NS5A interacts with FKBP38 and inhibits apoptosis in Huh7 hepatoma cells. *FEBS Lett* 2006; **580**: 4392-4400
 - 100 **Chung YL**, Sheu ML, Yen SH. Hepatitis C virus NS5A as a potential viral Bcl-2 homologue interacts with Bax and inhibits apoptosis in hepatocellular carcinoma. *Int J Cancer* 2003; **107**: 65-73
 - 101 **Lan KH**, Sheu ML, Hwang SJ, Yen SH, Chen SY, Wu JC, Wang YJ, Kato N, Omata M, Chang FY, Lee SD. HCV NS5A interacts with p53 and inhibits p53-mediated apoptosis. *Oncogene* 2002; **21**: 4801-4811
 - 102 **Street A**, Macdonald A, Crowder K, Harris M. The Hepatitis C virus NS5A protein activates a phosphoinositide 3-kinase-dependent survival signaling cascade. *J Biol Chem* 2004; **279**: 12232-12241
 - 103 **Sarcar B**, Ghosh AK, Steele R, Ray R, Ray RB. Hepatitis C virus NS5A mediated STAT3 activation requires co-operation of Jak1 kinase. *Virology* 2004; **322**: 51-60
 - 104 **Gong G**, Waris G, Tanveer R, Siddiqui A. Human hepatitis C virus NS5A protein alters intracellular calcium levels, induces oxidative stress, and activates STAT-3 and NF-kappa B. *Proc Natl Acad Sci USA* 2001; **98**: 9599-9604
 - 105 **Nanda SK**, Herion D, Liang TJ. The SH3 binding motif of HCV [corrected] NS5A protein interacts with Bin1 and is important for apoptosis and infectivity. *Gastroenterology* 2006; **130**: 794-809
 - 106 **Macdonald A**, Harris M. Hepatitis C virus NS5A: tales of a promiscuous protein. *J Gen Virol* 2004; **85**: 2485-2502

S- Editor Ma N L- Editor Alpini GD E- Editor Ma WH

Effects and mechanisms of electroacupuncture at PC6 on frequency of transient lower esophageal sphincter relaxation in cats

Chi Wang, De-Feng Zhou, Xiao-Wei Shuai, Jian-Xiang Liu, Peng-Yan Xie

Chi Wang, De-Feng Zhou, Xiao-Wei Shuai, Jian-Xiang Liu, Peng-Yan Xie, Department of Gastroenterology, First Hospital of Peking University, Beijing 100034, China
Supported by the National Natural Science Foundation of China, No. C030503039

Correspondence to: Dr. Peng-Yan Xie, Department of Gastroenterology, Peking University First Hospital, 8 Xishiku street, Beijing 100034, China. xiepengyan@medmail.com.cn
Telephone: +86-10-66551122-2581 Fax: +86-10-66551580
Received: June 2, 2007 Revised: July 12, 2007

Abstract

AIM: To investigate the effects of electroacupuncture (EA) at neiguan (PC6) on gastric distention-induced transient lower esophageal sphincter relaxations (TLESRs) and discuss the mechanisms of this treatment.

METHODS: Protocol I: Twelve healthy cats underwent gastric distention for 60 min on the first day. Electrical acupoint stimulation was applied at the neiguan or a sham point on the hip in randomized order before gastric distention, on the third day and fifth day. Those cats that underwent EA at neiguan on the fifth day were named "Neiguan Group" and the cats that underwent EA at a sham acupoint on the fifth day were named "Sham Group" (control group). During the experiment the frequency of TLESRs and lower esophageal sphincter (LES) pressure were observed by a perfused sleeve assembly. Plasma levels of gastrin (GAS) and motilin (MTL) were determined by radioimmunoassay. Nitrite/nitrate concentration in plasma and tissues were measured by Griess reagent. The nuclei in the brain stem were observed by immunohistochemistry method of c-Fos and NADPH-d dyeing. Protocol II: Thirty six healthy cats were divided into 6 groups randomly. We gave saline (2 mL iv. control group), phaclofen (5 mg/kg iv. GABA-B antagonist), cholecystokinin octapeptide (CCK-8) (1 µg/kg per hour iv.), L-Arginine (200 mg/kg iv.), naloxone (2.5 µmol/kg iv.) and tacrine (5.6 mg/kg ip. cholinesterase inhibitor) respectively before EA at Neiguan and gastric distention. And the frequencies of TLESRs in experimental groups were compared with the control group.

RESULTS: Protocol I: Not only the frequency of gastric distention-induced TLESR in 60 min but also the rate of common cavity during TLESRs were significantly

decreased by EA at neiguan compared to that of sham acupoint stimulation. C-Fos immunoreactivity and NOS reactivity in the solitarius (NTS) and dorsal motor nucleus of the vagus (DMV) were significantly decreased by EA at neiguan compared to that of the sham group. However, the positive nuclei of C-Fos and NOS in reticular formation of the medulla (RFM) were increased by EA at neiguan. Protocol II: The inhibited effect of EA at neiguan on TLESRs frequency was completely restored by pretreatment with CCK (23.5/h vs 4.5/h, $P < 0.05$), L-arginine (17.5/h vs 4.5/h, $P < 0.05$) and naloxone (12/h vs 4.5/h, $P < 0.05$). On the contrary, phaclofen (6/h vs 4.5/h, $P > 0.05$) and tacrine (9.5/h vs 4.5/h, $P > 0.05$) did not influence it.

CONCLUSION: Electric acupoint stimulation at Neiguan significantly inhibits the frequency of TLESR and the rate of common cavity during TLESR in cats. This effect appears to act on the brain stem, and may be mediated through nitric oxide (NO), CCK-A receptor and mu-opioid receptors. But the GABAB receptor and acetylcholine may not be involved in it.

© 2007 WJG. All rights reserved.

Key words: Electroacupuncture; Transient lower esophageal sphincter relaxation; C-Fos; Nitric oxide; Cat

Wang C, Zhou DF, Shuai XW, Liu JX, Xie PY. Effects and mechanisms of electroacupuncture at PC6 on frequency of transient lower esophageal sphincter relaxation in cats. *World J Gastroenterol* 2007; 13(36): 4873-4880

<http://www.wjgnet.com/1007-9327/13/4873.asp>

INTRODUCTION

Transient lower esophageal sphincter relaxations (TLESRs) is the most important mechanism of gastroesophageal reflux (GER) either in the patients of gastroesophageal reflux disease (GERD)^[1] or in normal subjects^[2]. Distention of the proximal stomach is a major stimulus for triggering TLESRs^[3]. The stimulus passes through vago-vagal reflex and is integrated in the brain stem. There is ongoing interest in developing drugs that can decrease GER by interfering with TLESRs, including

GABA-B receptor agonist^[4], Cholecystokinin-A receptor antagonist^[5], nitric oxide synthase (NOS) inhibitor^[5,6], morphine^[7] and atropine (act through central cholinergic blockade)^[8-10]. The aim of our study is to explore new approaches (EA at Neiguan acupoint) to decrease the rate of TLESRs during gastric distention, and discuss the mechanisms of this treatment.

Acupuncture has been used to treat functional gastrointestinal disorders in eastern countries for centuries. It can modulate visceral sensation as well as function through stimulation at selected acupoints along the meridians (channels of acupoints)^[11]. EA at zusanli (ST-36) can increase the basal LES pressure^[12]. Transcutaneous electric nerve stimulation (TENS) at Hukou acupoint increased the degree of LES relaxation in volunteers^[13] and reduced basal LES pressure in patients with achalasia^[14,15]. Previous studies have suggested that TENS at neiguan may inhibit the rate of TLESRs triggered by gastric distention^[16] and reduce the perception to gastric distention^[17] in human beings. But the precise mechanisms for this phenomenon have not been extensively investigated and are not fully understood.

MATERIALS AND METHODS

Materials

Protocol I was performed on 12 adult cats weighing 3.7 ± 0.2 kg (M/F: 8/4), and 36 adult cats weighing 3.6 ± 0.5 kg (M/F: 25/11) were studied for protocol II. Cats were provided by the Animal Center of the First Hospital of Peking University. They were kept in individual cages in a controlled environment with a temperature of 22-26°C, 12/12-h light/dark cycles, and fed with standard cat diet. The animals were deprived of food 10 h before each experiment. All procedures were approved by the Committee for Animal Care and Usage for Research and Education of the Peking University. Anesthesia was initially induced with katamine (30 mg/kg im). Supplementary doses of katamine (15 mg/kg ip) were given whenever necessary to maintain an appropriate depth of anesthesia, as assessed they remained motionless yet still had cornea reflex. They were euthanized with pentobarbital sodium (0.5 mL/kg ip) at the end of the protocol.

Phaclofen, CCK octapeptide and tacrine were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA). Naloxone was provided from Beijing Shuanghe Chemical Company. L-arginine was offered by Beijing Dingguo Chemical Company.

The GAS Radioimmunoassay kit was purchased from the China Institute of Atomic Energy, Beijing, China. The MTL Radioimmunoassay kit was purchased from the Neurobiological Technique Center of the Second Military Medical University, Shanghai, China. The Griess reagent for testing nitrite/nitrate concentration was purchased from Promega Corporation, USA. The reduced form nicotinamide adenine dinucleotide phosphate (NADPH) of and NBT (nitro blue tetrazolium) were obtained from Biomol Corporation, London, UK. The antibody of C-Fos was obtained from Santa Cruz Biotechnology, California, USA.

Recording methods

The manometry catheter (outer diameter 0.5 cm) consisted of a multilumen silicone tube with five side holes located at 9, 6, 3, 0 and -6 cm from the upper margin of the 6 cm-long Dent sleeve sensor (Dentsleeve, Belair, Australia). The catheter was continuously perfused with distilled water by a low compliance pneumohydraulic capillary infusion system (UPS-2020, Holland) at a rate of 0.2 mL/min. The external pressures transducers were connected via an analog/digital converter to a personal computer system. The data were displayed continuously on a monitor and stored on the personal computer system (MMS B.V. the Netherlands).

After anesthesia the cat was set in a supine position. A manometry catheter was placed through the mouth into the esophagus and positioned so that the sleeve sensor straddled the LES to register LES pressure. The distal side hole was used as a reference point for intragastric pressure. And the upper LES side holes were used to measure esophageal body pressure.

A mylohyoid electromyography (MMS B.V. the Netherlands) was used to record swallowing^[11,18]. The pinhead electrode was inserted in the mylohyoid muscle, and the reference electrode was fixed to the interscapular region of the back.

Electroacupuncture

Two acupuncture needles of 0.22 mm in diameter (Suzhou global acupuncture instrument Co. Ltd, Suzhou, China) were inserted perpendicularly at the bilateral Neiguan acupoint (PC6, located 1.5-2.0 cm above the wrist between the ligaments of the flexor carpi radialis and the palmaris longus^[19]) overlying the median nerve to a depth of 5 mm. An electrical stimulator (Model LH202H Hans, Beijing Huawei Medical Instrument Co. Ltd, Beijing, China) provided current to the needles. Wave patterns were sparse with dense pulse intervals ranging from 2 to 100 Hz (2/100 Hz), with constant amplitude and current flow (3-4 mA). The duration was 60 min. Correct positioning was confirmed by observing slight repetitive paw flexion during stimulation^[20].

Control stimulation on a sham acupoint was conducted at the hip, a point away from the traditional meridians and dermatomes.

Gastric distention

Air was insufflated (at a rate of 15 mL/s) into the stomach through a 2.0-mm diameter tube intubated through mouth to stomach. It's depth equal to 5 cm plus the esophageal body length. 30 mL air into the stomach every 6 min amount to 300 mL were insufflated in the 1 h period of gastric distention.

Study protocol I : Effects of EA on LES motor function

Twelve adult cats were divided into Neiguan group and Sham group randomly. Each cat was studied for three sessions, and an interval of each session is 2 d (Figure 1). In the first session, the basal rate of TLESRs triggered by gastric distention was observed for one hour. In session 2 and session 3, the influence of EA at neiguan or sham

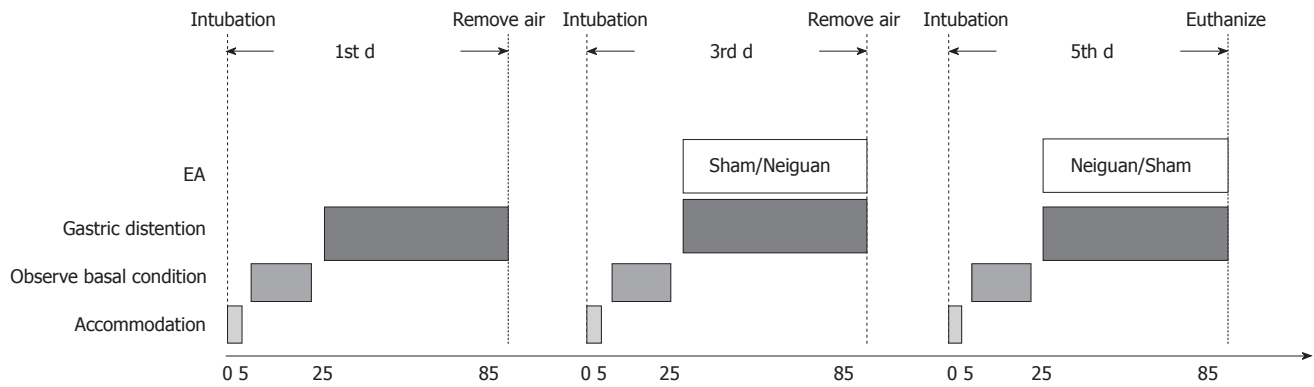


Figure 1 The flow chart of protocol I.

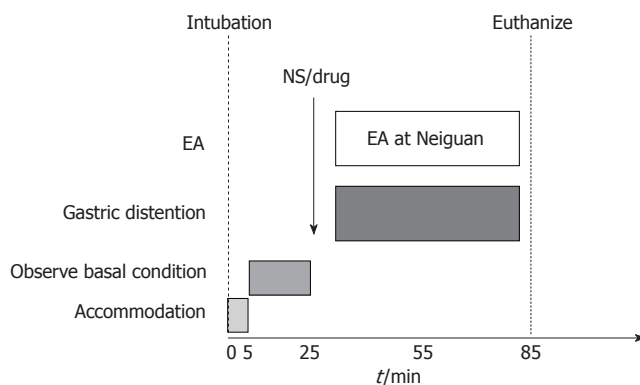


Figure 2 The process of protocol II.

acupoint to the rate of gastric distention induced TLESRs was observed in a random order.

After 5min of accommodation, the basal conditions were observed for 20 min (including basal rate of TLESRs and basal LES pressure). And then the gastric distention was applied in session 1. In session 2 and 3, gastric distention and electroacupuncture were applied at the same time. The remaining air was removed at the end of each session. At the end of session 3, the cats were euthanized and the blood, gastric fundus and brain stem were obtained for further research.

Study protocol I : Effects of drugs (phaclofen, CCK octapeptide, L-arginine, naloxone and tacrine) on inhibition of TLESRs by electric acupoint stimulation

Thirty six healthy cats were divided into 6 groups randomly. We gave normal saline(NS) (2 mL iv.), GABA-B antagonist phaclofen^[21] (5 mg/kg iv.), CCK octapeptide^[5] (1 µg/kg per hour iv.), L-arginine^[5] (200 mg/kg iv.), naloxone^[22] (2.5 µmol/kg iv.) and tacrine^[23] (5.6 mg/kg ip. cholinesterase inhibitor in central nerve system) respectively before EA at Neiguan and gastric distention, and observed the frequency of TLESR and LES pressure (Figure 2). At the end of this study, the cats were euthanized and the blood, gastric fundus and brain stem were obtained for further research.

Data analysis

TLESRs were defined according to established methods^[9].

Basal LES pressure was measured at the end of expiration relative to gastric pressure. The LES pressure during gastric distention was measured for 1 min every 6 min, and an overall mean for each period of the study was calculated. Common cavities were defined as abrupt simultaneous and sustained rises of basal esophageal pressure to intragastric pressure in at least the two lower esophageal body manometry recording sites^[24]. Common cavities are considered as markers of gas or liquid reflux from the stomach into the esophagus.

Assay of gastrointestinal hormones and nitrite concentration

When the cats were euthanized, 15 mL venous blood was collected into a test tube containing 400 µL of 10% EDTA-Na₂ (an anticoagulant) and 200 µL of trasytol. The blood samples were centrifuged at 4°C at 3500 r/min for 20 min. The serum was separated and stored at -70°C before analysis. The tissue of gastric fundus is about 2 cm × 2 cm. After weighting, tissues were added to 1 mL of 1 mol/L acetic acid and mixed evenly in a homogenizer to obtain a homogenate and were refrigerated at 4°C for 100 min. The extracted homogenates were added to 1 mL of 1 mmol/L NaOH and centrifuged at 3500 r/min 4°C for 20 min. The supernatant fluid was collected and stored at -70°C.

Gastrin (GAS) and motilin (MTL) levels were measured with commercial radioimmunoassay kits. Nitrite/nitrate levels were tested by Griess reagent.

c-Fos immunohistochemistry and NADPH-d histochemistry

After 60 min of gastric distention on the fifth day, the animal was transcardially perfused with 9 g/L saline followed by 40 g/L paraformaldehyde in 0.1 mol/L phosphate buffer saline (PBS, pH 7.3). The brain stem was removed and postfixed in the same fixative overnight and cryoprotected by immersion in 200 g/L sucrose for 72 h.

Coronal sections (40 µm) of the brain were cut in a cryostat. Every fourth section was used to reveal c-Fos immunoreactivity and the second set of sections was used to reveal NADPH-diaphorase (NADPH-d) staining.

The first set of sections were placed into a 50 g/L goat serum for 30 min at room temperature (RT), and incubated overnight at RT in primary antibody c-Fos (1:200). After washing for 15 min with PBST, the sections were incubated in biotinylated anti-rabbit IgG

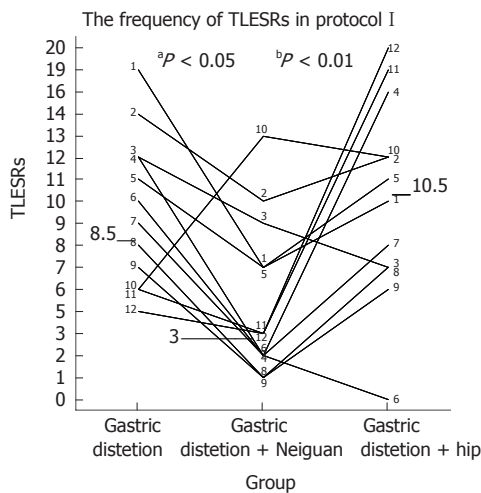


Figure 3 EA at neiguan decreased the rate of TLESRs induced by gastric distention, compared with EA at sham acupoint ($^aP < 0.01$) and baseline distention ($^bP < 0.05$). Each number represents one cat.

(Zymed, South San Francisco, Canada) diluted 1:300 in PBST at RT for 2 h, and then incubated in peroxidase-conjugated streptavidin (1:300 dilution, Zymed) for 2 h at RT. The immunoreactivity was visualized by incubating with 0.05 mol/L Tris-HCl buffer containing 0.1 g/L 3, 3'-diaminobenzidine, and 0.3 mL/L H_2O_2 for 10-20 min at RT. The stained sections were mounted on APES-coated glass slides, dehydrated and coverslipped.

The second set of sections were incubated at 37°C for 2 h in a solution containing 1 mmol/L NADPH, 0.5 mmol/L NBT, Tris-HCl 50 mmol/L, and Triton X-100 2 g/L. After a rinse in PBST, sections were mounted on APES-coated glass slides, dehydrated and coverslipped.

The distribution of c-Fos and NADPH-d positive cells was detected under a microscope (Olympus, Tokyo, Japan), and the cells were counted on LEICA Q550CW system (Leica Microsystems Imaging Solutions Ltd, Wetzlar, Germany), 10 sections for NTS/DMV, 8 sections for RFM. The average number of c-Fos or NADPH-d positive neurons per section for each cat was calculated, respectively.

Statistical analysis

The number of TLESRs was compared using Wilcoxon signedrank test and expressed as median (interquartile range). Basal LES pressure and intragastric pressure were presented as means \pm SD and were compared using repeated-measures. The rate of common cavity is compared using paired sample χ^2 . Nitrate concentrations, plasma hormone levels and the average number of c-Fos or NADPH-d positive nucleus of respective brain areas per section were expressed as mean \pm SD and was compared using independent t .

SPSS 11.0 was used for statistical analysis, and $P < 0.05$ was considered statistically significant.

RESULTS

Study protocol I: Effects of EA on LES motor function

Transient LES relaxations: The frequency of TLESRs

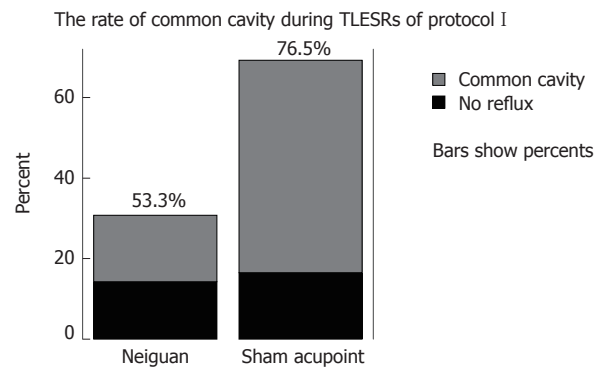


Figure 4 EA at Neiguan decreased the rate of common cavity during TLESRs compared with EA at sham acupoint ($P < 0.05$).

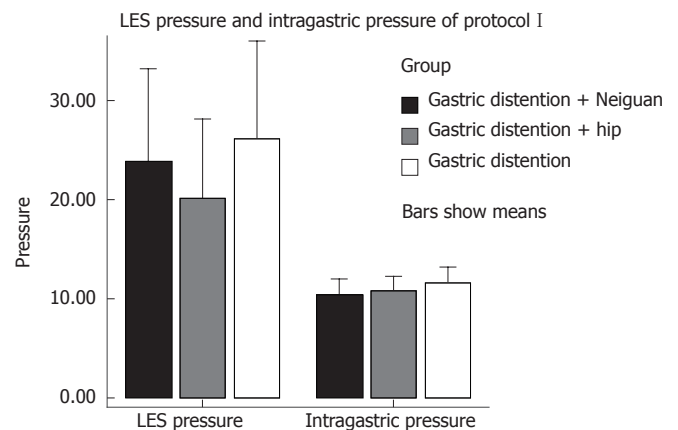


Figure 5 Electric stimulation at Neiguan didn't influence the LES pressure and intragastric pressure.

during acupoint stimulation at neiguan [3 per hour (range, 1-13)] was significantly lower than that during both the baseline period without any stimulation [8.5 per hour (range, 5-19), $P < 0.05$] and the period of sham stimulation at the hip [10.5 per hour (range, 0-20), $P < 0.01$] (Figure 3).

Common cavities during TLESRs: During EA at neiguan, a total of 60 TLESRs induced by gastric distention were observed in one hour, from it 32 were associated with common cavity, and its rate was 53.3%. During EA at sham acupoint, there are 136 gastric distention induced TLESRs in all, and 104 were associated with common cavity. The rate was 76.5%. Between-group comparisons showed that the rate of common cavity during EA at Neiguan was significantly lower than that occurring during EA at Sham acupoint ($P < 0.05$) (Figure 4).

LES pressure: EA at neiguan (PC6) had no effect on LES pressure. Overall mean LES pressure during electrical acupoint stimulation at neiguan (34.33 ± 18.16 mmHg) was similar to that during stimulation at the sham acupoint (30.97 ± 15.72 mmHg, $P > 0.05$) and during the baseline period without any acupoint stimulation (37.74 ± 18.69 mmHg, $P > 0.05$) (Figure 5).

Intragastric pressure: EA at neiguan (PC6) had no effect on gastric pressure during gastric distention. Overall

Table 1 Nitrite concentration in plasma and gastric fundus of protocol I (mean \pm SD)

Group	Serum (mmol/mL)	Gastric fundus (mmol/mg)
Neiguan	0.85 \pm 0.62	3.50 \pm 0.87
Sham	0.66 \pm 0.59	4.18 \pm 1.09

No significant difference was found between the two groups.

Table 2 Plasma GAS and MTL in each group of protocol I (mean \pm SD)

Group	GAS (pg/mL)	MTL (pg/mL)
Neiguan group	79.43 \pm 28.84	82.23 \pm 43.79
Sham group	156.30 \pm 72.53	103.6 \pm 68.16

GAS: gastrin; MTL: motilin. No significant difference was found between the two groups.

Table 3 c-Fos and NADPH-d staining in CNS

group	C-Fos		NOS	
	NTS and DMV	RFM	NTS and DMV	RFM
Neiguan group	21.9 \pm 6.9 ^a	96.6 \pm 16.5 ^a	23.6 \pm 4.6 ^a	75.6 \pm 17.0 ^a
Sham group	45.3 \pm 10.1	61.0 \pm 13.8	43.9 \pm 11.0	30.6 \pm 8.5

NADPH: nicotinamide adenine dinucleotide phosphate; CNS: central nerve system; NOS: nitric oxide synthase; NTS: nucleus tractus solitarius; DMV: dorsal motor nucleus of vagus; RFM: reticular formation of medulla. ^a*P* < 0.05 vs Sham group.

mean gastric pressure during EA at neiguan (10.37 \pm 3.17 mmHg) was similar to that during stimulation at the sham acupoint (10.81 \pm 2.89 mmHg, *P* > 0.05) and during the baseline period without any acupoint stimulation (11.58 \pm 3.16 mmHg, *P* > 0.05) (Figure 5).

Nitrite concentration: EA at neiguan (PC6) did not influence the nitrite levels in plasma and gastric fundus tissues compared with Sham group (*P* > 0.05) (Table 1).

Gastrin (GAS) and motilin (MTL) levels: EA at neiguan (PC6) did not influence the GAS and MTL levels in plasma (Table 2).

C-Fos and NADPH-d staining in the central nerve system (CNS)

The c-Fos positive cell nuclei of activated cells showed the characteristic dark brown staining of oxidized DAB. Compared with the Sham group, electroacupuncture at neiguan significantly inhibited the number of C-Fos-labeled neurons in nucleus tractus solitarius/dorsal motor nucleus of vagus (NTS/DMV) (Figure 6A and B). However, it stimulated a significantly greater number of C-Fos positive nuclei in areas of reticular formation of the medulla (RFM) (Figure 7A and B).

NADPH-d activity was visualized as a vibrant blue color within perikarya, dendrites and axons. Electroacupuncture at neiguan significantly decreased the number of

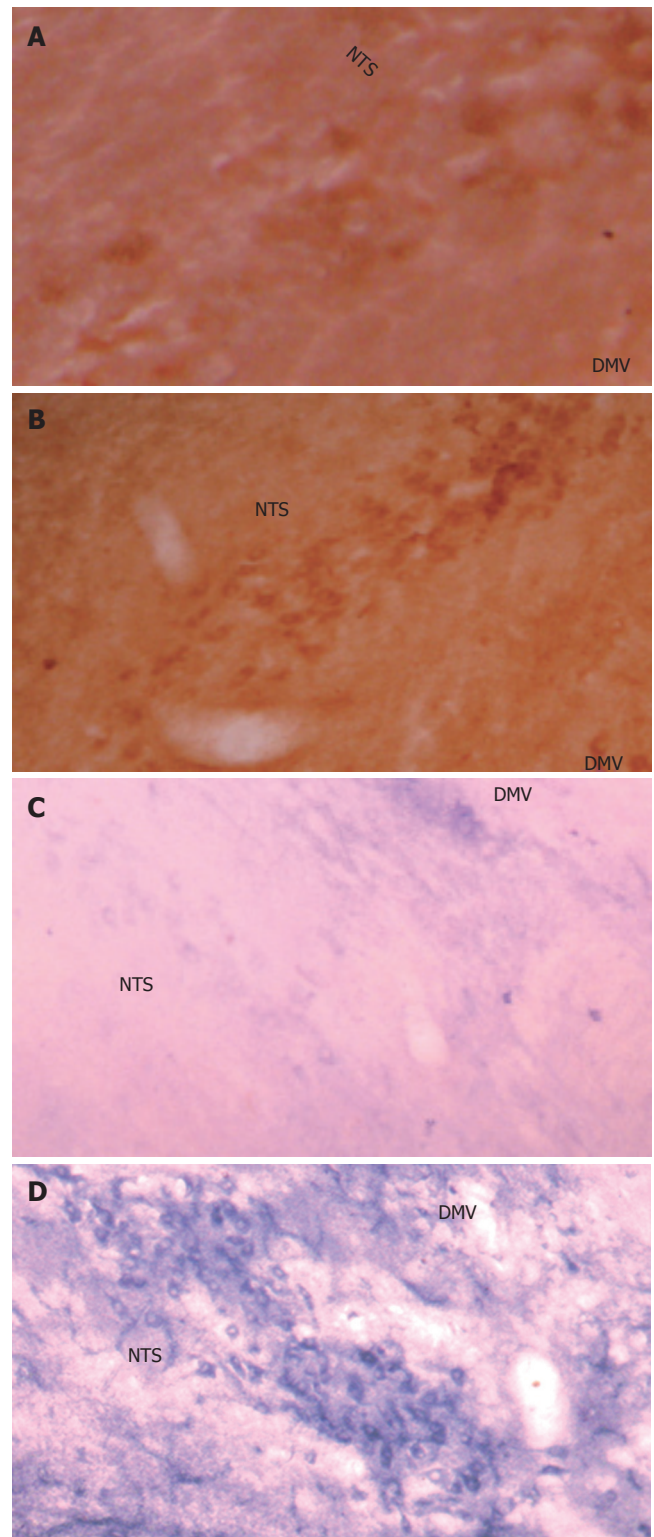


Figure 6 Photomicrographs showing C-Fos and NOS positive neurons in nucleus tractus solitarius/dorsal motor nucleus of vagus (NTS/DMV). A: C-Fos immunohistochemistry of neiguan group; B: C-Fos immunohistochemistry of sham acupoint group; C: NADPH-d histochemistry of neiguan group; D: NADPH-d histochemistry of sham acupoint group (\times 10).

NADPH-d stained cells in NTS/DMV (Figure 6C and D), but increased the number of positive cells in RFM (Figure 7C and D).

The number of positive nuclei in each group is listed in Table 3.

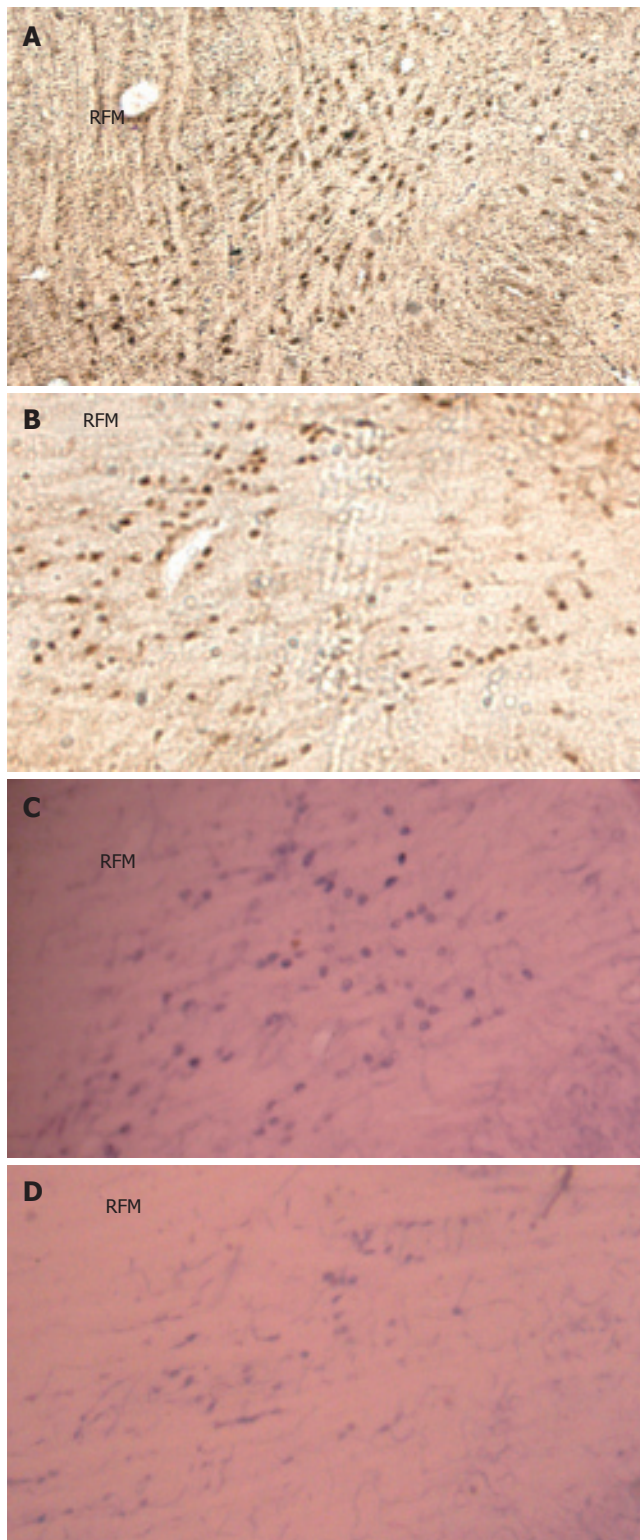


Figure 7 Photomicrographs showing C-Fos and NOS positive neurons in reticular formation of medulla (RFM). **A:** C-Fos immunohistochemistry of neiguan group; **B:** C-Fos immunohistochemistry of sham acupoint group; **C:** NADPH-d histochemistry of neiguan group; **D:** NADPH-d histochemistry of sham acupoint group ($\times 10$).

Study Protocol II : Effects of Drugs (phaclofen, CCK octapeptide, L-arginine, naloxone and tacrine) on inhibition of TLESRs by Electric Acupoint Stimulation

Transient LES relaxations: After saline infusion, the frequency of TLESRs during acupoint stimulation at neiguan [4.5 per hour (range, 1-11)] was still significantly

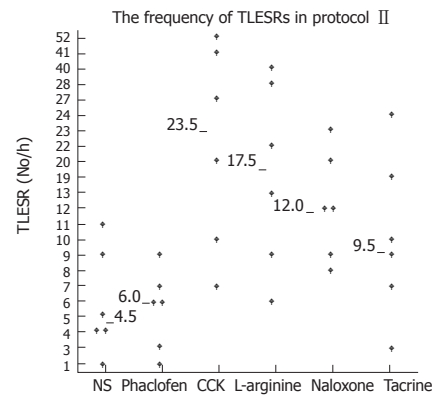


Figure 8 CCK, L-arginine and naloxone significantly increase the frequency of TLESRs compared with control group ($^{\circ}P < 0.05$). But phaclofen and tacrine did not change the frequency of TLESRs compared with control group ($P > 0.05$). It was indicated that the inhibited effects of EA at neiguan were completely restored by pretreatment with CCK-A, L-arginine and naloxone. But it was not influenced by phaclofen and tacrine.

lower than that during sham stimulation at the hip in protocol I [10.5 per hour (range, 0-20), $P < 0.05$].

Infusion of Phaclofen and tacrine did not influence the rate of TLESRs during acupoint stimulation at neiguan. They were [6 per hour (range 1-9) *vs* 4.5 per hours (range 1-11), $P > 0.05$] and [9.5 per hour (range 3-24) *vs* 4.5 per hour (range, 1-11), $P > 0.05$] respectively. However, during infusion of CCK octapeptide, L-arginine and naloxone, the inhibited effect of EA at neiguan had been completely restored. The rate of TLESRs were CCK [23.5 per hour (range 7-52) *vs* 4.5 per hours (range 1-11), $P < 0.05$], L-arginine [17.5 per hour (range 6-40) *vs* 4.5 per hours (range 1-11), $P < 0.05$] and naloxone [12 per hour (range 8-23) *vs* 4.5 per hours (range 1-11), $P < 0.05$] respectively (Figure 8).

DISCUSSION

Acupuncture has been used to treat functional gastrointestinal disorders in the eastern countries for centuries. A large amount of clinical evidence supports the effectiveness of acupuncture for treating functional disorders of the gastrointestinal tract, and the most commonly used acupoints in treating gastrointestinal symptoms are Neiguan (PC6) and Zusanli (ST-36). In the present study, we found electric acupoint stimulation at the neiguan acupoint resulted in a significant reduction of the rate of TLESRs induced by gastric distention. This result was consistent with previous investigations on human beings [12]. Furthermore, the present work also demonstrated the action site and neurotransmitters of this effect.

Speculation on the action site of electroacupuncture at neiguan

We postulate that the site at which electric acupuncture stimulation acts to inhibit the occurrence of TLESRs may be as follows: First, it may increase the proximal gastric motility or increase the gastric fundus tone, so that to decrease the volume of gastric fundus, and inhibit

the stretch receptors localized in the gastric fundus (As we know, the stretch receptors were the major receptor in triggering TLESRs^[25,26]) and then reduce the sensory input from gastric distention. Second, it may inhibit the integration of TLESRs in some area of the brain stem, such as NTS (nucleus tractus solitarius) and DMV (dorsomotor nucleus of the vagus nerve). Third, electric acupoint stimulation at Neiguan may exert its action primarily on the efferent motor pathway.

With present research, electric stimulation at Neiguan did not change the intragastric pressure and nitrite levels in the tissue of gastric fundus compared with EA at sham acupoint. Nitric oxide is well accepted as an inhibitory neurotransmitter in the gastrointestinal tract, and may exert a tonic inhibition on the proximal stomach^[27]. So the result of our study suggested that EA at neiguan cannot change the tone of gastric fundus. And it is consistent with previous research^[12,28].

In the current research, electric stimulation at neiguan had no effect on the residual LES pressure, so it is unlikely that it exerts action primarily on the efferent motor pathway.

The dorsal vagal complex (DVC) comprising nucleus tractus solitarius (NTS) and dorsomotor nucleus of the vagus nerve (DMV) is the center of the integration of TLESRs^[9]. The brain stem dyeing shows that when compared with sham group EA at neiguan significantly decreases the C-Fos and NOS positive nucleus in NTS/DMV. However, it stimulated a significantly greater number of C-Fos and NOS positive nucleus in areas of reticular formation of the medulla (RFM). And RFM may be one of the acupuncture action sites^[29].

Consequently, the action site of EA at neiguan may be localized within the brain stem. It may increase NOS in the nucleus of RFM, so that it inhibits NOS in NTS/DMV, and then decreases the frequency of TLESRs.

Speculation on the neurotransmitter of electroacupuncture at neiguan

In the second part of this research, the inhibited effect of EA at neiguan on TLESR's rate was completely restored by pretreatment with CCK, L-arginine and naloxone. On the contrary, phaclofen and tacrine did not influence it. So this effect appeared to be mediated through nitric oxide (NO), CCK-A receptor and mu-opioid receptors. But the GABAB receptor and acetylcholine may not be involved in it.

In our study, electric stimulation at Neiguan also did not influence the gastrin and motilin levels in plasma. It suggested that the inhibited effects of electric stimulation were not through these two neuropeptides.

The variance between previous research

Endogenous opioid peptides (EOPs) are considered as major candidates for a role in acupuncture action because numerous investigations have clearly demonstrated that electroacupuncture effect is antagonized by the opioid receptor antagonist naloxone^[30]. And in our research, the naloxone can reverse the inhibited effect of electroacupuncture on TLESRs. In contrast to our results in cats, Zou *et al.*^[12] found the inhibited effect of

acupoint stimulation was not inhibited by naloxone. In that study, the frequency of electrical stimulation is 100 Hz. However, our wave patterns were sparse and dense pulse intervals ranging from 2 Hz to 100 Hz (2/100 Hz). Previous studies have demonstrated that low-frequency (2 Hz) electroacupuncture analgesia (EAA) is induced by the activation of μ - and δ -opioid receptors *via* the release of enkephalin, β -endorphin, and endomorphin; and high-frequency (100 Hz) EAA is caused by activation of κ opioid receptors *via* release of dynorphin^[31]. So it may be the reason of the variance between our findings and Zou's research.

In conclusion, electric acupoint stimulation at the Neiguan result in a significant reduction of the rate of TLESRs induced by gastric distention. This effect appears to act at the brain stem, and may be mediated through NO, CCK-A receptor and mu-opioid receptor.

COMMENTS

Background

Gastroesophageal reflux disease (GERD) is a disorder characterized by an increased exposure of the esophagus to the intragastric contents. Recent studies have suggested that transient lower esophageal sphincter relaxation is the main mechanism underlying gastroesophageal reflux. It involves a prolonged relaxation of the lower esophageal sphincter, mediated by a vago-vagal neural pathway, synapsing in the brainstem. Acupuncture has been used to treat functional gastrointestinal disorders in the eastern countries for centuries. It can modulate visceral sensation as well as function through stimulation at selected acupoints along the meridians (channels of acupoints).

Research frontiers

Transient lower esophageal sphincter relaxation (TLESR) is the most important mechanism of gastroesophageal reflux (GER) in the patients of GERD. So it had become an important target in dealing with gastroesophageal reflux disease.

Innovations and breakthroughs

Although there have one article indicated that electroacupuncture at neiguan (PC6) may decrease the frequency of TLESRs, our research is the first paper to observe the mechanisms carefully.

Applications

Our research observed the relationship between electroacupuncture at neiguan (PC6) and TLESRs. And there may be a significant clinical impact in the future.

Terminology

TLESR: It is a spontaneous relaxation of LES without swallow induced. Its definition included: (1) absence of swallowing for 4 s before to 2 s after the onset of LES relaxation. (2) relaxation rate of $> \text{or} = 1 \text{ mmHg/s}$. (3) time from onset to complete relaxation of $< \text{or} = 10 \text{ s}$. (4) nadir pressure of $< \text{or} = 2 \text{ mmHg}$. Exception: a markedly prolonged LES relaxation $> \text{or} = 10 \text{ s}$, and nadir pressure $< \text{or} = 2 \text{ mmHg}$ can also be classified as TLESR, irrespective of the relation with swallow; Common Cavity: It was defined as abrupt simultaneous and sustained rises of basal esophageal pressure to intragastric pressure in at least the two lower esophageal body manometry recording sites.

Peer review

This manuscript it is well written and the experimental design is sound. In addition, there may be a significant clinical impact if these results will be confirmed in the future.

REFERENCES

- 1 Mittal RK, McCallum RW. Characteristics and frequency of transient relaxations of the lower esophageal sphincter in

- patients with reflux esophagitis. *Gastroenterology* 1988; **95**: 593-599
- 2 **Dent J**, Dodds WJ, Friedman RH, Sekiguchi T, Hogan WJ, Arndorfer RC, Petrie DJ. Mechanism of gastroesophageal reflux in recumbent asymptomatic human subjects. *J Clin Invest* 1980; **65**: 256-267
 - 3 **Holloway RH**, Hongo M, Berger K, McCallum RW. Gastric distention: a mechanism for postprandial gastroesophageal reflux. *Gastroenterology* 1985; **89**: 779-784
 - 4 **Lee KJ**, Vos R, Janssens J, Tack J. Differential effects of baclofen on lower oesophageal sphincter pressure and proximal gastric motility in humans. *Aliment Pharmacol Ther* 2003; **18**: 199-207
 - 5 **Boulant J**, Fioramonti J, Dapoigny M, Bommelaer G, Bueno L. Cholecystokinin and nitric oxide in transient lower esophageal sphincter relaxation to gastric distention in dogs. *Gastroenterology* 1994; **107**: 1059-1066
 - 6 **Hirsch DP**, Tiel-Van Buul MM, Tytgat GN, Boeckstaens GE. Effect of L-NMMA on postprandial transient lower esophageal sphincter relaxations in healthy volunteers. *Dig Dis Sci* 2000; **45**: 2069-2075
 - 7 **Penagini R**, Allocca M, Cantù P, Mangano M, Savojardo D, Carmagnola S, Bianchi PA. Relationship between motor function of the proximal stomach and transient lower oesophageal sphincter relaxation after morphine. *Gut* 2004; **53**: 1227-1231
 - 8 **Fang JC**, Sarosiek I, Yamamoto Y, Liu J, Mittal RK. Cholinergic blockade inhibits gastro-oesophageal reflux and transient lower oesophageal sphincter relaxation through a central mechanism. *Gut* 1999; **44**: 603-607
 - 9 **Mittal RK**, Holloway RH, Penagini R, Blackshaw LA, Dent J. Transient lower esophageal sphincter relaxation. *Gastroenterology* 1995; **109**: 601-610
 - 10 **Lidums I**, Hebbard GS, Holloway RH. Effect of atropine on proximal gastric motor and sensory function in normal subjects. *Gut* 2000; **47**: 30-36
 - 11 **Li Y**, Tougas G, Chiverton SG, Hunt RH. The effect of acupuncture on gastrointestinal function and disorders. *Am J Gastroenterol* 1992; **87**: 1372-1381
 - 12 **Mu XD**, Xie PY, Liu JX, Shuai XW, Li J. Effect of Electroacupuncture at Zusanli acupoint on LESP, plasma gastrin and motilin in rats. *Shijie Huaren Xiaohua Zazhi* 2005; **13**: 1069-1073
 - 13 **Chang FY**, Chey WY, Ouyang A. Effect of transcutaneous nerve stimulation on esophageal function in normal subjects-evidence for a somatovisceral reflex. *Am J Chin Med* 1996; **24**: 185-192
 - 14 **Guelrud M**, Rossiter A, Souney PF, Sulbaran M. Transcutaneous electrical nerve stimulation decreases lower esophageal sphincter pressure in patients with achalasia. *Dig Dis Sci* 1991; **36**: 1029-1033
 - 15 **Kaada B**. Successful treatment of esophageal dysmotility and Raynaud's phenomenon in systemic sclerosis and achalasia by transcutaneous nerve stimulation. Increase in plasma VIP concentration. *Scand J Gastroenterol* 1987; **22**: 1137-1146
 - 16 **Zou D**, Chen WH, Iwakiri K, Rigda R, Tippet M, Holloway RH. Inhibition of transient lower esophageal sphincter relaxations by electrical acupoint stimulation. *Am J Physiol Gastrointest Liver Physiol* 2005; **289**: G197-G201
 - 17 **Coffin B**, Azpiroz F, Malagelada JR. Somatic stimulation reduces perception of gut distention in humans. *Gastroenterology* 1994; **107**: 1636-1642
 - 18 **Mittal RK**, McCallum RW. Characteristics of transient lower esophageal sphincter relaxation in humans. *Am J Physiol* 1987; **252**: G636-G641
 - 19 **Tjen-A-Looi SC**, Li P, Longhurst JC. Prolonged inhibition of rostral ventral lateral medullary premotor sympathetic neurons by electroacupuncture in cats. *Auton Neurosci* 2003; **106**: 119-131
 - 20 **Grossi L**, Ciccaglione AF, Travaglini N, Marzio L. Transient lower esophageal sphincter relaxations and gastroesophageal reflux episodes in healthy subjects and GERD patients during 24 hours. *Dig Dis Sci* 2001; **46**: 815-821
 - 21 **Han Y**, Qing J, Bu DF. Gamma-aminobutyric acid B receptor regulates the expression of hydrogen sulfide/cystathionine- β -synthase system in recurrent febrile seizures. *Zhonghua Dangdai Erke Zazhi* 2006; **4**: 141-143
 - 22 **Gustafsson BI**, Delbro DS. Vagal influence on the motility of the feline jejunum. *J Physiol* 1994; **480** (Pt 3): 587-595
 - 23 **Nielsen JA**, Mena EE, Williams IH, Nocerini MR, Liston D. Correlation of brain levels of 9-amino-1,2,3,4-tetrahydroacridine (THA) with neurochemical and behavioral changes. *Eur J Pharmacol* 1989; **173**: 53-64
 - 24 **Holloway RH**, Wyman JB, Dent J. Failure of transient lower oesophageal sphincter relaxation in response to gastric distension in patients with achalasia: evidence for neural mediation of transient lower oesophageal sphincter relaxations. *Gut* 1989; **30**: 762-767
 - 25 **Penagini R**, Carmagnola S, Cantù P, Allocca M, Bianchi PA. Mechanoreceptors of the proximal stomach: Role in triggering transient lower esophageal sphincter relaxation. *Gastroenterology* 2004; **126**: 49-56
 - 26 **Tack J**, Sifrim D. A little rest and relaxation. *Gut* 2000; **47**: 11-12
 - 27 **Shen GM**, Zhou MQ, Xu GS, Xu Y, Yin G. Role of vasoactive intestinal peptide and nitric oxide in the modulation of electroacupuncture on gastric motility in stressed rats. *World J Gastroenterol* 2006; **12**: 6156-6160
 - 28 **Li P**, Rowshan K, Crisostomo M, Tjen-A-Looi SC, Longhurst JC. Effect of electroacupuncture on pressor reflex during gastric distension. *Am J Physiol Regul Integr Comp Physiol* 2002; **283**: R1335-R1345
 - 29 **Miller AD**. Central mechanisms of vomiting. *Dig Dis Sci* 1999; **44**: 39S-43S
 - 30 **Sodipo JO**, Gilly H, Pauser G. Endorphins: mechanism of acupuncture analgesia. *Am J Chin Med* 1981; **9**: 249-258
 - 31 **Han JS**. Acupuncture and endorphins. *Neurosci Lett* 2004; **361**: 258-261

S- Editor Zhu LH L- Editor Li M E- Editor Ma WH



Impaired contractility and remodeling of the upper gastrointestinal tract in diabetes mellitus type-1

Jens Brøndum Frøkjær, Søren Due Andersen, Niels Ejsskjær, Peter Funch-Jensen, Asbjørn Mohr Drewes, Hans Gregersen

Jens Brøndum Frøkjær, Søren Due Andersen, Asbjørn Mohr Drewes, Center for Visceral Biomechanics and Pain, Aalborg Hospital, Aalborg, Denmark

Jens Brøndum Frøkjær, Department of Radiology, Aalborg Hospital, Aalborg, Denmark

Niels Ejsskjær, Department of Endocrinology M, Aarhus University Hospital, Aarhus, Denmark

Peter Funch-Jensen, Department of Surgical Gastroenterology L, Aarhus University Hospital, Aarhus, Denmark

Asbjørn Mohr Drewes, Hans Gregersen, Center for Sensory-Motor Interaction, Department of Health Science and Technology, Aalborg University, Denmark

Hans Gregersen, National Center for Ultrasound in Gastroenterology, Haukeland University Hospital, Bergen, Norway

Correspondence to: Jens Brøndum Frøkjær, MD, PhD, Center for Visceral Biomechanics and Pain, Department of Radiology, Aalborg Hospital, DK-9100 Aalborg, Denmark. jf@mech-sense.com
Telephone: +45-99326825

Received: February 20, 2007 Revised: March 21, 2007

Abstract

AIM: To investigate that both the neuronal function of the contractile system and structural apparatus of the gastrointestinal tract are affected in patients with longstanding diabetes and autonomic neuropathy.

METHODS: The evoked esophageal and duodenal contractile activity to standardized bag distension was assessed using a specialized ultrasound-based probe. Twelve type-1 diabetic patients with autonomic neuropathy and severe gastrointestinal symptoms and 12 healthy controls were studied. The geometry and biomechanical parameters (strain, tension/stress, and stiffness) were assessed.

RESULTS: The diabetic patients had increased frequency of distension-induced contractions (6.0 ± 0.6 vs 3.3 ± 0.5 , $P < 0.001$). This increased reactivity was correlated with the duration of the disease ($P = 0.009$). Impaired coordination of the contractile activity in diabetic patients was demonstrated as imbalance between the time required to evoke the first contraction at the distension site and proximal to it (1.5 ± 0.6 vs 0.5 ± 0.1 , $P = 0.03$). The esophageal wall and especially the mucosa-submucosa layer had increased thickness in the patients ($P < 0.001$), and the longitudinal and radial compressive stretch was less in diabetics ($P <$

0.001). The esophageal and duodenal wall stiffness and circumferential deformation induced by the distensions were not affected in the patients (all $P > 0.14$).

CONCLUSION: The impaired contractile activity with an imbalance in the distension-induced contractions likely reflects neuronal abnormalities due to autonomic neuropathy. However, structural changes and remodeling of the gastrointestinal tract are also evident and may add to the neuronal changes. This may contribute to the pathophysiology of diabetic gut dysfunction and impact on future management of diabetic patients with gastrointestinal symptoms.

© 2007 WJG. All rights reserved.

Key words: Diabetes; Autonomic Neuropathy; Biomechanics; Contractility; Ultrasound; Esophagus; Duodenum; Deformation; Stress

Frøkjær JB, Andersen SD, Ejsskjær N, Funch-Jensen P, Drewes AM, Gregersen H. Impaired contractility and remodeling of the upper gastrointestinal tract in diabetes mellitus type-1. *World J Gastroenterol* 2007; 13(36): 4881-4890

<http://www.wjgnet.com/1007-9327/13/4881.asp>

INTRODUCTION

Gastrointestinal (GI) symptoms (nausea, vomiting, bloating, abdominal pain, diarrhea, etc.) are frequent in patients with diabetes mellitus^[1-4]. The symptoms are often severe and substantially compromise quality of life. Abnormal GI function in diabetic patients has been demonstrated with methods such as manometry, scintigraphy, radiography, and breath tests. For example, the esophagus is characterized by dysmotility with fewer contractions having decreased amplitudes and abnormal wave forms^[5]. The pathogenesis of the GI symptoms in diabetes is complex in nature, multi-factorial and not well-understood^[5]. Dysmotility and delayed emptying of the stomach have been demonstrated, and in the small and large intestine dysmotility, delayed transit, and bacterial overgrowth have been observed^[5]. The GI dysfunction and symptoms may be caused by autonomic neuropathy being one of the most prevalent complications affecting

up to 40% of patients with long-standing diabetes^[6,7], and several clinical studies have demonstrated neuropathy of the autonomic nervous system (especially vagal but also sympathetic), as well of enteric nerves^[8]. Impaired visceral sensory function, glycemic control and psychological factors may also be contributing factors^[9-13]. Finally, mechanical factors may also contribute to the symptoms. Hence, studies in animals with experimental diabetes have shown structural remodeling and protein cross-linking in the GI wall layers compared to control animals^[14-18]. Structural remodeling caused by diabetes in animals is known to cause changes in the biomechanical properties, resulting in increase of both stiffness and thickness of the GI wall^[19-22].

The impact of the structural changes in the human GI wall on the function and on biomechanical properties has not been studied in detail due to inaccessibility of the organs and lack of suitable methodology. Such studies are needed since it is still generally assumed that the contractile (and structural) apparatus of the GI tract is normal, and that the disordered function and abnormal contractile activity predominantly reflects neuronal abnormalities^[23,24]. Better ways of studying the GI tract may impact on the future management of diabetic patients with GI symptoms. Cross-sectional ultrasound imaging have recently been developed to study the biomechanical properties of the GI tract during distension in animals^[25,26] and humans^[27-29]. The deformation pattern, the radial distributions of strain, stress and stiffness, and the distension-induced sensation have been assessed in the human esophagus^[30]. The technique has also been applied to assess the biomechanical properties of the human duodenum^[31]. Ultrasound has no known short or long term hazards and provides excellent soft tissue imaging with a good temporal and spatial resolution and is a valuable tool for studying GI function *in vivo*.

The hypotheses in the present study were that (1) the biomechanical properties of the esophageal and duodenal wall were changed due to diabetes-induced tissue remodeling and that (2) the contractile activity of the esophagus and duodenum was affected by the neuronal dysfunction related to diabetic autonomic neuropathy. Hence, the aims were to apply the new ultrasound based testing approach to (1) assess the distension-induced contractile activity in the human upper gut in healthy controls and in patients affected by gastrointestinal dysfunction due to diabetic autonomic neuropathy, and (2) to look into the mechanism of the findings by assessing the GI remodeling including the wall thickness and distension-induced deformation patterns.

MATERIALS AND METHODS

Study subjects

Data were obtained from 15 diabetic patients recruited at the Department of Endocrinology M, Aarhus University Hospital (13 males, 2 females, mean age 43 years, range 25-62 years) and 12 healthy controls (7 males, 5 females, mean age 37 years, range 29-50 years) recruited among the hospital staff and at the university. The local Ethical Committee approved the study protocol (VN

2003/120mch) which also conforms to the Declaration of Helsinki. Oral and written informed consent was obtained from all subjects.

All of the 12 patients who completed the study (Table 1) had type 1 diabetes lasting 12 to 46 years (average 23 years) and all suffered from debilitating symptomatic diabetic autonomic neuropathy (shown by a minimum of two symptoms from different organ systems) and verified by abnormal cardiovascular reflexes (heart rate variability and blood pressure changes during deep breathing and going from lying to standing). All suffered from peripheral neuropathy as demonstrated by absent or diminished patellar reflexes and abnormal biotensimetry values. The patients underwent examinations that were justified by their symptoms to exclude any organic diseases affecting the GI tract. Clinical data from the 12 patients who completed the study are presented below and in Table 1. All patients had severe GI symptoms: nausea (12 of 12), vomiting (10 of 12), abdominal pain (4 of 12), diarrhea (9 of 12), and constipation (2 of 12). Five of the 12 patients were taking medication known to affect gastrointestinal function (erythromycin, metoclopramide and proton pump inhibitors) while the rest were not treated because of previous insufficient response to various drugs. Four patients suffered from neuropathic pain and were treated with analgesics (oxycodone, gabapentin, pregabalin and paracetamol). None of the patients had prior abdominal surgery or suffered from psychiatric diseases or had any suspicion of psychological abnormalities. The control subjects did not take medications, had no prior abdominal surgery and did not suffer from any GI symptoms or pain-related diseases. They all had normal physical examination and blood tests.

Experimental probe design

The probe consisted of a 120 cm catheter (Ditens A/S, Aalborg, Denmark) with a 6.2 mm outer diameter and eight lumens of different sizes (Figure 1 top)^[30]. A 50 μ m thick polyurethane bag (Ditens A/S, Aalborg, Denmark) was attached to the catheter with 5 cm between the attachment points and with its centre positioned corresponding to the crystal of the ultrasound probe. The bag could be inflated to a maximum diameter of 50 mm (cross-sectional area (CSA) of 2000 mm²) with a constant bag length without stretching the bag wall. The size of the bag was chosen on the basis of previous studies of the duodenum where the CSA never exceeded 2000 mm² when the bag was inflated to the point where moderate pain was reported^[32].

The largest lumen in the probe contained a 20 MHz endoscopic 360 degrees ultrasound probe (UM-3R, Olympus Corporation, Tokyo, Japan). The signal from the endoscopic ultrasound unit (EU-M30, Olympus Corporation, Tokyo, Japan) was directly captured and stored digitally (AVI MPEG4 format) by frame grabber software (Studio 8, Pinnacle Systems Inc., CA, USA) for later analysis. Another large lumen was for infusion and withdrawal of fluid to the bag. The lumen was connected to a roller pump (Type 110, Ole Dich, Hvidovre, Denmark) for inflation and deflation of the bag with 37°C sterile water at a constant rate^[30,33].

Two small lumens (< 1 mm in diameter) were used for

Table 1 Clinical data describing the 12 type-1 diabetic patients that completed the study

Patient No.	1	2	3	4	5	6	7	8	9	10	11	12
Gender	M	M	F	M	M	M	M	M	M	M	M	F
Age (yr)	30	47	25	32	40	46	42	39	41	62	53	33
Height (cm)	176	180	165	176	169	182	180	185	168	174	175	170
Weight (kg)	64	86	65	60	90	78	75	85	66	79	95	61
Diabetes duration (yr)	18	37	16	12	32	27	32	14	13	46	20	14
Autonomic neuropathy	S,T	S,T	S,T	S,T	S,T	S	S,T	S	S	S	S	S,T
Peripheral neuropathy	S,T	S,T	S	S,T	S,T	S	S,T	S	S	S	S	S,T
Neuropathic pain	-	+	-	-	-	-	+	+	-	-	-	+
Retinopathy	+	+	+	+	+	+	+	+	+	+	+	+
Nephropathy	-	+	+	-	+	+	+	-	-	+	+	+
Bladder paresis	-	-	-	-	+	-	-	-	-	-	-	+
Gastroparesis ¹	+	+	+	+	+	-	-	-	-	+	-	+
Sexual dysfunction	-	-	-	-	-	-	-	-	+	-	-	+
HbA _{1c} (%)	11.1	8.6	14.1	10.5	7.7	10.7	9.2	9.3	10.4	10.2	9.2	7.3
Creatinine (mmol/L)	75	101	111	63	73	87	123	78	96	108	118	52
Nausea	+	+	+	+	+	+	+	+	+	+	+	+
Vomiting	+	+	+	+	+	-	+	+	+	+	+	-
Abdominal pain	+	-	+	-	+	-	-	-	-	+	-	-
Diarrhea	+	-	+	+	+	+	+	+	+	-	-	+
Constipation	-	-	-	-	+	-	-	+	-	-	-	-
Gastroscopy	N	N	N	N	N	N	N	N	N	N	N	N
Colonoscopy	ND	ND	N	ND	N	ND	ND	N	N	ND	ND	N
Small bowel radiology	ND	ND	N	N	N	N	ND	ND	N	N	ND	N
Breath test ²	N	ND	N	N	N	N	ND	ND	ND	N	ND	N
Insulin treatment	inj	pump	inj	inj	inj	inj	inj	inj	inj	inj	inj	pump
GI medication	-	-	+	-	-	-	+	-	+	+	-	+
Analgetics	-	+	-	-	-	-	+	+	-	-	-	+
Smoking	+	-	+	+	-	+	-	+	-	+	-	-

M: male, and F: female. S: verified by classic symptoms, and T = verified by tests. “-”: not present, and “+”: present. N: normal examination, and ND: not done. “inj”: injection by insulin pen. “pump”: injection by pump system. ¹Assessed by scintigraphy. ²To exclude bacterial overgrowth. Normal range of HbA_{1c} is < 6% and Creatinine < 125 mmol/L (M) or < 115 mmol/L (F).

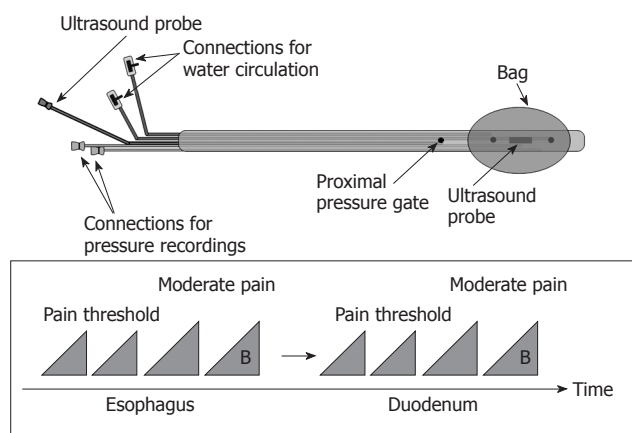


Figure 1 The probe design allows bag distension of the esophagus and duodenum together with cross-sectional ultrasound imaging and recording of the bag and proximal pressures (top). The distension protocol is shown at the bottom. The first distensions to the pain threshold were preconditioning stimuli also used for learning. They were followed by a distension (15 mL/min in the esophagus and 25 mL/min in the duodenum) to the perception of moderate pain. Finally, a bolus of 20 mg of butylscopolamine (B) was given intravenously in order to diminish distension-evoked smooth muscle contractions and a final distension to the perception of moderate pain was done.

recording of pressures inside the bag and 6 cm proximal to the center of the bag. The channels were continuously perfused at a rate of 0.1 mL/min with sterile water by a low-compliance perfusion system. The pressure channels were attached to external pressure transducers (Baxter,

Deerfield, IL, United States). The signals were amplified, analogue-to-digital converted and stored on a computer for later analysis (Openlab, Ditens A/S, Aalborg, Denmark).

Study protocol

The patients were fasting for 12 h prior to the experiment due to the well known delayed gastric emptying in this patient group (Table 1). During the fasting period the blood glucose concentrations were monitored every hour and adjusted to approximate the normal range (below 6 mmol/L) using intravenous glucose infusions and subcutaneous injection of fast-acting insulin (Actrapid, Novo, Bagsværd, Denmark). The healthy controls were fasting for six hours (Figure 1, bottom).

The probe was swallowed and the subject was positioned supine with the upper part of the body 30 degrees tilted. The lower esophageal sphincter was identified guided by the pressure recordings and the ultrasound image. The bag was positioned 10 cm above the lower esophageal sphincter. Distension was done by inflating the bag at a rate of 15 mL/min. Several preconditioning distensions to the pain threshold were done prior to experimental distensions to the perception of moderate pain^[34,35]. The preconditioning distensions were repeated until the obtained data were reproducible. The bag was deflated between distensions at a rate of 15 mL/min. Then 20 mg of butylscopolamine (Buscopan, Boehringer, Ingelheim, Germany) was administrated intravenously to abolish esophageal contractility followed

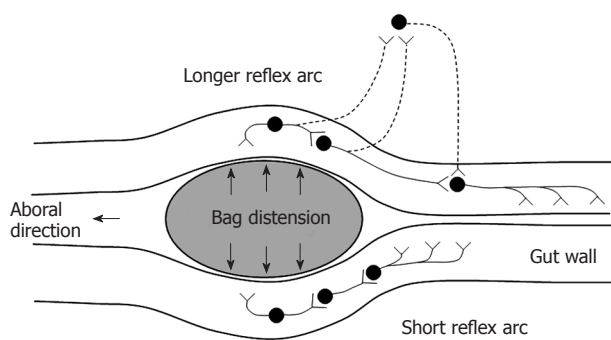


Figure 2 Schematic illustration of the neural pathways involved in distension-induced contraction. The primary afferent neurons, the excitatory interneurons and efferent neurons are shown. The dotted neurons illustrate extrinsic pathways. The induction of contraction on the bag depends on the local reflex arc (bottom). The measurement 6 cm proximal to the bag depends on a longer reflex arc including extrinsic pathways (top).

by one distension to the perception of moderate pain.

The probe was then advanced into the horizontal part of the duodenum guided by transabdominal ultrasound (SonoSite 180, SonoSite Inc, Bothell, WA, USA), endoscopic ultrasound and by the motility pattern observed. Approximately 30 min after the esophageal testing, and when duodenal phase II activity was observed, duodenal distensions were performed before and after administration of butylscopolamine as described above but at an inflation rate of 25 mL/min.

The total examination time was approximately two hours and the blood glucose concentrations in all patients and controls were measured before, after one hour, and at the end of the study. The blood glucose level was adjusted to approximate the normal range if it deviated during the study.

Analysis of the distension-induced contractile activity

Contractions were defined as having amplitude ≥ 10 cm H₂O and duration ≥ 3 s. Contractile activity at the distension site and proximal to it were analyzed for the filling phase of all distensions. The time from start of the distension to induction of the first contraction during the distension procedure was noted. The time to induce the first contraction at the distension site is generally thought to be dependent on a local short enteric reflex arc. The time to induce the first contraction 6 cm proximal to the bag depends on a longer reflex arc that is more likely affected by extrinsic pathways (Figure 2). Hence, the ratio between these contractile responses serves as a proxy of the function of the neural pathway between the bag and proximal pressure measurement site. The number of contractions during the first minute and the frequency of all contractions were also noted. Furthermore, the pressure amplitude and duration of the strongest contraction were calculated. A proxy of the contractile work was computed by multiplying the amplitude and the duration of the strongest contraction. In the duodenum the frequency of contractions at the proximal pressure measurement site was also calculated before and after the distension procedure.

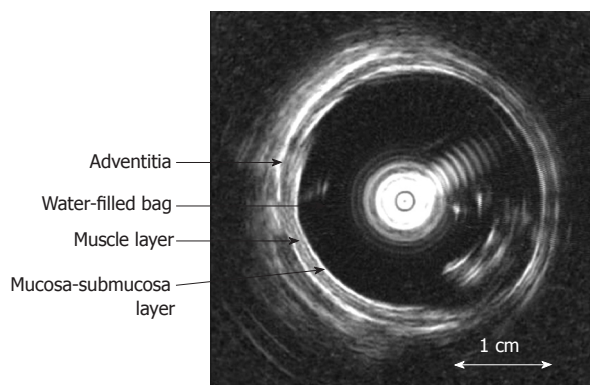


Figure 3 The cross-sectional ultrasound image of the distended distal esophagus allows identification of the esophageal layers, i.e. mucosa-submucosa, muscle and adventitia layers. The white round shadow in the centre is caused by the intraluminal ultrasound probe.

Biomechanical analysis of esophageal and duodenal distensions

The biomechanical properties including stress-strain and tension-strain relations, esophageal wall thickness and multidirectional deformation were measured and calculated from the intraluminal ultrasound images (Figure 3) and pressure recordings (see Appendix).

Statistical analysis

Data are given as mean \pm SE unless otherwise stated. The contractile activity of the patients and controls was analyzed using one-way analysis of variance (ANOVA). The contraction frequencies in the duodenum in relation to the distension and the duodenal wall stiffness were analyzed using two-way ANOVA, which was also used for the esophageal stress, strain, stress-strain ratio, multidirectional deformation and wall thickness, and duodenal tension, strain and tension-strain ratio at maximum perception. The esophageal wall stiffness was analyzed using three-way ANOVA with the factors: (1) controls *vs* patients, (2) before *vs* after butylscopolamine, and (3) mucosal surface *vs* submucosa-muscle interface *vs* outer surface. The association between the clinical data and the contractile activity and biomechanical properties was analyzed using Spearman correlation test. *P*-values less than 0.05 were considered significant. If the data were not normal-distributed they were logarithmically transformed before the parametric tests were performed. SPSS version 11.0 was used for the statistical analysis.

RESULTS

The study was completed in 12 (10 males, 2 females, mean age 40 years, range 25-62 years) of the 15 diabetic patients. Three patients interrupted the study in its initial phase due to severe throat irritation, nausea and vomiting. All healthy controls completed the study.

Blood glucose levels

At the beginning of the 12 h fasting period the diabetic patients had a mean blood glucose level of 10.6 (range

Table 2 The contractile activity on the bag and at the proximal pressure recording site are given for both the esophagus and duodenum (mean \pm SEM)

	Esophagus				Duodenum			
	Bag		Proximal		Bag		Proximal	
	Controls	Diabetes	Controls	Diabetes	Controls	Diabetes	Controls	Diabetes
Time to first contraction (s)	17.5 \pm 6.0 <i>F</i> = 3.8	9.9 \pm 2.7 <i>P</i> = 0.07	35 \pm 9.0 <i>F</i> = 16	6.6 \pm 2.3 <i>P</i> < 0.001	16.5 \pm 5.9 <i>F</i> = 0.4	15.9 \pm 5.2 <i>P</i> = 0.6	28 \pm 8 <i>F</i> = 2.2	6.5 \pm 2.5 <i>P</i> = 0.16
Ratio bag/proximal, time to first contraction	0.5 \pm 0.1 <i>F</i> = 5.8	1.5 \pm 0.6 <i>P</i> = 0.03			0.6 \pm 0.2 <i>F</i> = 3.6	1.2 \pm 0.2 <i>P</i> = 0.08		
Number of contractions in first minute	2.6 \pm 0.5 <i>F</i> = 14	5.9 \pm 0.7 <i>P</i> = 0.001	2.2 \pm 0.6 <i>F</i> = 5.8	4.5 \pm 0.8 <i>P</i> = 0.03	1.6 \pm 0.4 <i>F</i> = 0.2	1.9 \pm 0.5 <i>P</i> = 0.7	1.2 \pm 0.2 <i>F</i> = 2.9	2.1 \pm 0.7 <i>P</i> = 0.09
Frequency (min ⁻¹)	3.3 \pm 0.5 <i>F</i> = 13	6.0 \pm 0.6 <i>P</i> = 0.001	3.0 \pm 0.7 <i>F</i> = 2.6	4.6 \pm 0.8 <i>P</i> = 0.11	1.7 \pm 0.4 <i>F</i> = 0.03	1.7 \pm 0.5 <i>P</i> = 0.9	1.2 \pm 0.3 <i>F</i> = 0.5	1.7 \pm 0.4 <i>P</i> = 0.5
Maximum amplitude (mm H ₂ O)	95 \pm 11 <i>F</i> = 4.9	63 \pm 10 <i>P</i> = 0.04	37 \pm 7 <i>F</i> = 0.09	34 \pm 5 <i>P</i> = 0.8	19.1 \pm 4.8 <i>F</i> = 0.01	19.5 \pm 5.1 <i>P</i> = 0.9	15.9 \pm 2.6 <i>F</i> = 0.08	14.6 \pm 3.8 <i>P</i> = 0.8
Contractile work (mm H ₂ O x s)	1470 \pm 257 <i>F</i> = 7.8	592 \pm 165 <i>P</i> = 0.01	386 \pm 57 <i>F</i> = 7.5	181 \pm 43 <i>P</i> = 0.01	106 \pm 50 <i>F</i> = 0.9	88 \pm 43 <i>P</i> = 0.8	60 \pm 15 <i>F</i> = 0.6	42 \pm 16 <i>P</i> = 0.4

P- and *F*-values are given, and bold *P*-values indicate significant difference between the diabetes patients and healthy controls.

7.0-16.5) mmol/L with fluctuations during the first hours. The glucose level was adjusted and stabilized to a mean level of 8.1 (range 5.2-10.5) mmol/L during the study period. The controls had a mean blood glucose level (after 6 h fasting) of 4.6 (range 3.5-5.2) mmol/L during the study period.

Distension-induced contractile activity

Data on the contractile activity induced by the bag distensions are provided in Table 2.

Esophagus: The diabetic patients had shorter time to the first contraction on the bag (borderline), increased number of bag contractions during the first minute, increased frequency of contractions, reduced pressure amplitudes and reduced contractile work of the bag contractions. Similar findings were found 6 cm proximal to the bag where the diabetic patients had shorter time to the first contraction, increased number of contractions during the first minute, and reduced contractile work of the contractions. The ratio between the time until the first contraction on the bag and the time until the first contraction 6 cm proximal to the bag was increased in the diabetic patients. This likely reflects dysfunction of local intestinal neural pathways.

Duodenum: The contractile activity between the diabetic patients and controls did not differ in the duodenum. The ratio between the time until the first contraction on the bag and the time until the first proximal contraction tended to increase in the diabetic patients. Furthermore, the frequency of contractions in the diabetic duodenum was 2.3 ± 1.0 min⁻¹ before, 2.1 ± 0.8 min⁻¹ during and 2.8 ± 0.6 min⁻¹ after the distension and in the controls the numbers were 1.5 ± 0.4 min⁻¹, 1.6 ± 0.6 min⁻¹ and 1.9 ± 0.8 min⁻¹, respectively. Hence, no difference between the patients and controls (*F* = 0.8, *P* = 0.4) and in relation to the distension (*F* = 0.5, *P* = 0.6) were found.

Esophageal wall thickness

The thickness of the entire esophageal wall, the muscle

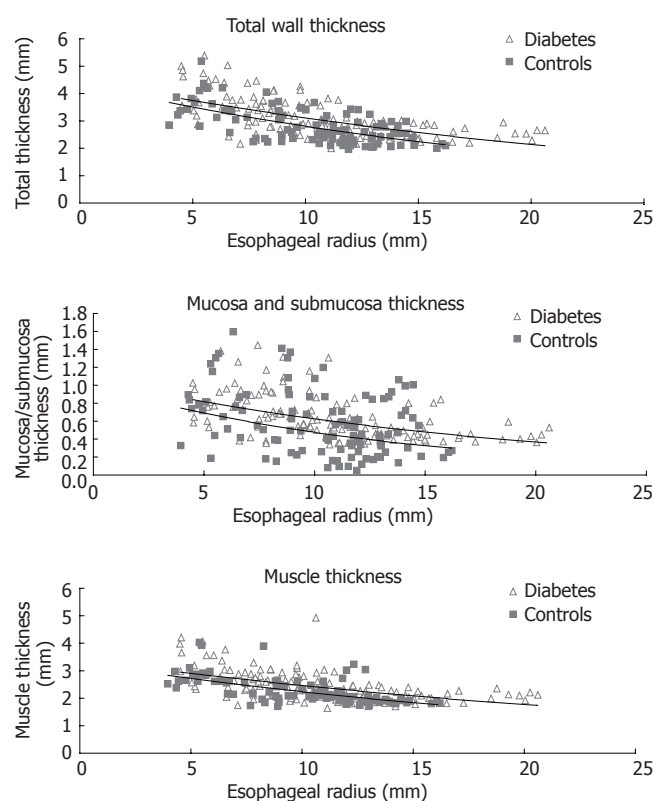


Figure 4 The distension-induced change in thicknesses of the total esophageal wall structure, the muscle layer, and mucosa-submucosa layer during smooth muscle relaxation with butylscopolamine are illustrated as function of the esophageal radius. The data points represent the multiple measuring points during each distension of the patients and controls. Exponential trend lines (solid lines) of the patients and controls are shown. The total wall thickness and the mucosa-submucosa layer were increased in the diabetic patients.

layer and mucosa-submucosa layer obtained during the distensions (after administration of butylscopolamine) are illustrated in Figure 4. The wall and the mucosa-submucosa layer were thicker (0.2-0.3 mm) in the diabetic patients compared to the control subjects (*F* = 13, *P* < 0.001, and *F* = 13, *P* < 0.001). The muscle layer thickness showed a borderline increase (*F* = 3.7, *P* = 0.055).

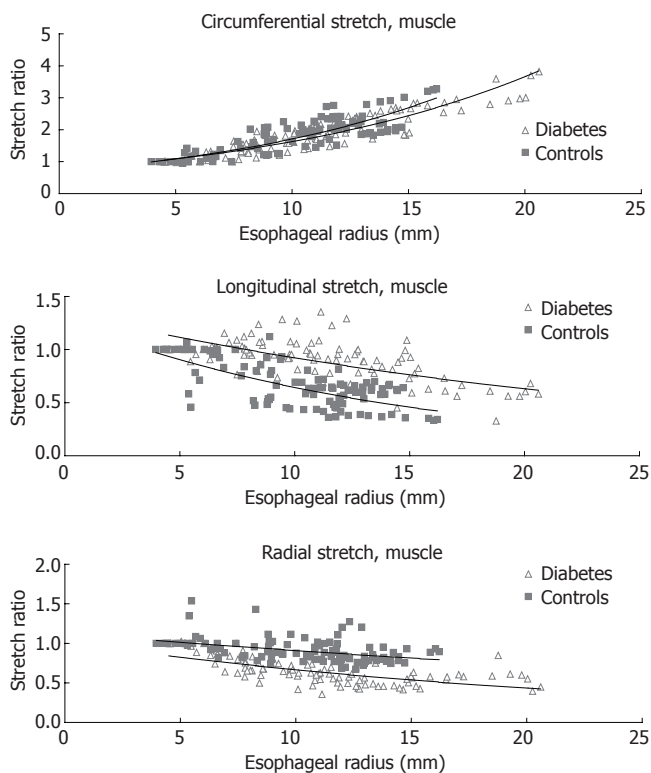


Figure 5 The distension-induced change in circumferential, longitudinal and radial stretch ratios are illustrated as function of the esophageal radius. The curves were obtained during smooth muscle relaxation with butylscopolamine. The data points represent the multiple measuring points during each distension of the patients and controls. Exponential trend lines (solid lines) of the patients and controls are shown. The shortening during distension was clearly reduced in the diabetic patients while the radial stretch was decreased.

Multidirectional deformation of the esophagus

The circumferential, longitudinal and radial stretch ratios during distension in both the diabetic patients and control subjects are shown in Figure 5. The curves were obtained from the muscle layer and after administration of butylscopolamine. The same pattern was seen in all sub-layers both before and after administration of butylscopolamine. During distensions the patients tended to stretch less in the circumferential direction ($F = 0.007$, $P = 0.1$). The compressive deformation (shortening) in the longitudinal and radial directions during distension was clearly reduced in the diabetic patients ($F = 150$, $P < 0.001$ for longitudinal direction and $F = 180$, $P < 0.001$ for the radial direction). Thus, the esophageal wall appeared to be deformed less in all normal directions in diabetics compared to healthy volunteers.

Stress-strain and tension-strain relations

The tissue stiffness (approximated by the mechanical alpha-constant, see Appendix) in circumferential direction of the different esophageal layers and the duodenum are provided in Figure 6.

Esophagus: The circumferential stiffness increased throughout the esophageal wall ($F = 14$, $P < 0.001$). The difference in tissue stiffness between the patients and the control subjects was non-significant ($F = 0.3$, $P = 0.6$) and unaffected by butylscopolamine ($F = 2.2$, $P = 0.15$).

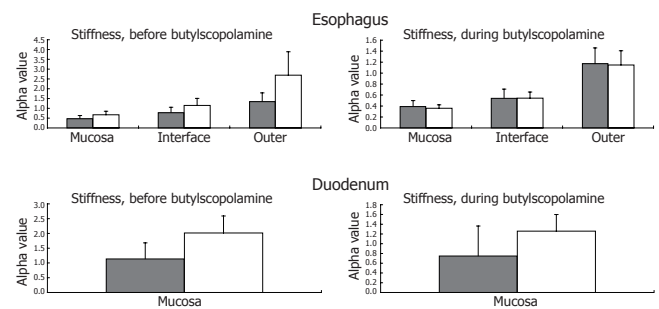


Figure 6 The computed esophageal stiffness (circumferential [alpha] constant) of the mucosa, submucosa-muscle interface and outer surface are illustrated. The solid grey bars represent the controls, while the grey-white bars represent the diabetic patients. The stiffness tends to be higher in the diabetic patients and is normalized during smooth muscle relaxation with butylscopolamine. This indicates an increased esophageal resting tone in diabetes. The computed duodenal stiffness is also illustrated. Mean and SEM values are shown.

At moderate induced pain, the circumferential stress, the circumferential strain and the ratio between them (which also indicates the wall stiffness) obtained in the patients were not significantly different from those obtained in the control subjects ($F = 0.7$, $P = 0.4$; and $F = 1.3$, $P = 0.3$; and $F = 0.7$, $P = 0.4$), and unaffected by butylscopolamine (all $P > 0.4$).

Duodenum: The difference in tissue stiffness (Figure 6) between the patients and the controls was non-significant ($F = 2.2$, $P = 0.14$). The stiffness was not changed by butylscopolamine ($F = 1.1$, $P = 0.3$). At moderate pain the circumferential tension, the circumferential strain and the ratio between them (indicating the wall stiffness) did not differ between patients and control subjects ($F = 1.0$, $P = 0.3$; and $F = 0.02$, $P = 0.9$; and $F = 2.3$, $P = 0.14$), and was unaffected by butylscopolamine (all $P > 0.5$).

Correlation to clinical data

The distension induced contractile activity in the diabetic patients was not affected by the mean glucose level during the study ($P > 0.2$ for all comparisons). The disease duration was clearly associated with the distension-induced contractile activity. Thus, the disease duration correlated with increased frequency of the contractions and with the number of contractions during the first minute proximal to the esophageal bag (correlation coefficient $r_s = 0.71$, $P = 0.009$ and $r_s = 0.69$, $P = 0.01$), and to increased duration of the strongest contraction on the esophageal bag ($r_s = 0.71$, $P = 0.009$). In the duodenum the disease duration correlated with increased frequency (bag: $r_s = 0.58$, $P = 0.04$; proximal: $r_s = 0.68$, $P = 0.01$), increased number of contractions during the first minute (bag: $r_s = 0.57$, $P = 0.04$; proximal: $r_s = 0.68$, $P = 0.04$), increased pressure amplitude (bag: $r_s = 0.59$, $P = 0.04$; proximal: $r_s = 0.70$, $P = 0.01$), duration (bag: $r_s = 0.62$, $P = 0.03$; proximal: $r_s = 0.69$, $P = 0.01$) and contractile work (bag: $r_s = 0.63$, $P = 0.03$; proximal: $r_s = 0.72$, $P = 0.01$) of the strongest contraction both at and proximal to the bag.

DISCUSSION

An ultrasound based testing approach was applied to

assess the contractile activity, geometry and biomechanical properties of the esophagus and duodenum in patients affected by gastrointestinal symptoms and diabetic autonomic neuropathy. Overall, this study illustrates that the patients had increased reactivity to standardized esophageal distension including dyscoordination and reduced contractile work of the contractions. The disease duration was associated with increased contractile reactivity. Finally, the esophageal wall thickness and the pattern of deformation in longitudinal and radial directions were affected in diabetic patients indicating remodeling.

Both acute and chronic hyperglycemia is known to impair the GI motor responses to stimulation^[12,36] and to reduce the perceived sensation^[37]. This study was designed to minimize the influence of the blood glucose level on the contractile activity. However, the patients in the present study typically suffered from severe fluctuating glucose levels and fluctuations in GI symptoms with intermittent nausea and vomiting which makes glycemic control very challenging. Even though the mean blood glucose level during the study was higher in the diabetic patients, the glucose level itself did not seem to affect the contractile activity. Hence, the differences found in this study are more likely due to neuromuscular changes. However, comparison of data obtained in different studies of diabetic patients must be done with caution.

The present study utilizes a *cross-sectional imaging technique*. Conventional methods based on *pressure-volume measurements* obtained during bag distension do not directly measure variables used in analysis of tissue deformation and stiffness^[38]. Since the esophagus is thick-walled, only cross-sectional ultrasound imaging with good temporal and spatial resolution provides data for computation of the circumferential stress, wall thickness and multi-directional deformation^[30].

All patients included in the study had, as per inclusion criteria, severe and long-lasting upper GI symptoms (nausea, vomiting, bloating and pain). The observed esophageal contractile hyperreactivity may be explained by both primary neuropathic changes of the gut nerves and secondary changes due to reflex mechanisms caused by increased sensitivity to the distensions (central sensitization)^[39]. However, esophageal motor abnormalities in terms of decreased contraction amplitude, decreased number of esophageal peristaltic contractions^[13,23,24,40] and impaired coordination^[41,42] have also been recorded in diabetic patients without GI symptoms. Disordered esophageal motility and acid reflux may be related to diabetic neuropathy^[43]. Such changes correspond to the changes observed during distension in the present study. The observed reduction in time until the first contraction on the bag in the diabetic patients suggests that a local neuronal dysfunction is responsible for the hyperreactivity. This is consistent with the finding that the hyperreactivity is associated with the disease duration. A neuronal dysfunction can theoretically be addressed to the mechanoreceptor, afferent fibers, interneurons, or efferent fibers located in the gut wall. Impaired balance of the inhibitory and excitatory pathways from the central nervous system can potentially also affect the motor response. The time until the first contraction 6 cm proximal to the bag was also reduced in the diabetic

patients, which may indicate hyperactivity and dysfunction of local intestinal neural pathways (a long reflex arc which may also include extra-intestinal pathways, Figure 2). Since this long reflex arc is more affected than the short arc (evidenced in Table 2 and by the computed ratio), the neuronal pathways rather than the mechanoreceptors seem to be affected. Even though neuropathic damage to the nerve fibers is expected, central (and peripheral) neuronal hyperexcitability in response to the distensions may counteract the response. Such hyperreactivity and hyperexcitability have for example been shown in patients with non-cardiac chest pain^[44]. In contrast, in structural GI disorders such as systemic sclerosis, the local mechanoreceptors in the gut wall seem to be affected (resetting) more than the neuronal pathways^[45]. However, the present study cannot definitively distinguish between neuronal changes restricted to the enteric nervous system and the effect of changes in the central inhibitory and excitatory neuronal pathways. Advanced methods using evoked brain potentials and functional magnetic resonance imaging may shed more light on this important aspect. Also one should take into consideration that the neuronal changes observed could be due to both diabetes-induced autonomic neuropathy and changes in the afferent visceral nervous system (sensitization and hypersensitivity) evoked by the long-standing GI symptoms^[39]. Finally, the vagal innervation of different organs and central *vs* peripheral levels can easily be affected to different degrees^[46]. As an indicator of dysfunction of the esophageal neuromuscular apparatus, the diabetic patients also seem to have an increased resting tone of the muscle component in the esophagus. This is indicated by the mechanical constants in Figure 6 where smooth muscle relaxation is shown to abolish/normalize the increased stiffness observed in the diabetic patients.

Studies of the small intestinal contractile activity in diabetes have revealed a wide spectrum of motor patterns ranging from normal to grossly abnormal^[5]. The distension-induced duodenal contractile activity recorded in this study was not clearly affected in diabetes compared to the control subjects. However, the duodenal reactivity increased with the disease duration.

The question is if the disordered contractile activities observed in these studies are only due to the neuronal changes and dysfunction (diabetes-induced autonomic neuropathy) or if primary diabetes-induced remodeling in the GI tract may also play a role. Animal studies have shown that diabetes may induce crypt hyperplasia, change in the villous microvasculature and increase in the mucosal and muscle mass^[14,17,19,47]. A histopathological study of the human stomach in diabetic patients with severe gastroparesis showed prominent collagenization and smooth muscle atrophy of the muscle layer^[48]. Studies on diabetes and aging show that advanced glycation end-products are causing cross linking of collagen molecules responsible for basement membrane thickening and loss of matrix elasticity^[15,16,49]. Animal studies support the presence of structural and biomechanical changes with increased stiffness, weight per unit length and wall thickness (i.e. increased stiffness and thickness of the GI wall)^[19-22]. Even though the wall stiffness and circumferential deformation induced by the circumferential distensions

in the present study were not significantly affected in diabetes, the esophageal deformations in longitudinal and radial directions and the wall thickness were abnormal with reduced deformation. The fact that the deformation is reduced but the stiffness appears unchanged can be attributed to the stress-dependent growth law for soft tissues stating that tissue remodeling is determined by the stress and remodels towards a tissue-specific stress level^[35]. The esophageal wall and the mucosa-submucosa layer were thickened in diabetes, which indicates growth processes of the intestinal wall. The observed increase in esophageal wall thickness can theoretically be caused by increased muscle tone, even though an attempt to abolish smooth muscle contractions was done and repeated if contractions were still present. Butylscopolamine diminishes cholinergic mediated tone and from the tracings it appeared that relaxation was obtained. Also the decreased ability of esophageal shortening during distension and the change in radial deformation supports that structural changes occur in diabetic esophagus. The contractile work of the esophageal contractions was decreased in spite of increased neuronal activity, increased muscle thickness and the increased esophageal resting tone. This indicates that the ability of the muscle to contract could be restricted by structural remodeling (accumulation of connective tissue). The fact that the esophageal wall deformed less in diabetic patients supports this hypothesis. Alternatively, myopathic abnormalities with diabetes-induced reduced action of the muscle fibers may also be important^[48].

The present study shows that both the neuronal function of the contractile system and structural apparatus of the GI tract are affected in patients with long-standing diabetes and autonomic neuropathy. This may contribute to the understanding of the pathophysiology of diabetic gut dysfunction and may have impact on future management of these patients.

ACKNOWLEDGMENTS

The Danish Health Research Council (SSVF), The Danish Diabetes Association, the Research Council of North Jutland County, the Toyota Foundation and the SparNord Foundation are acknowledged for funding this project.

APPENDIX

Analysis of esophageal distensions

Ultrasound images were captured corresponding to the start of the distension (the zero-pressure state) and between contractions during the distensions (tonic state) by a video converter (jpg format, 787 × 576 pixels, A4 Video Converter v. 2.3, www.a4video.com)^[35]. The mucosal surface (bag-mucosa interface), the submucosa-muscle interface, and the outer surface (muscle-adventitia interface) contours of the esophageal wall were identified by visual inspection by an experienced radiologist using image measurement software (SigmaScan Pro v. 5.0.0, SPSS Inc., Chicago, IL, USA) (Figure 3). This allowed computation of the cross-sections encircled by the mucosal surface (CSA_{muc}), submucosa-muscle interface (CSA_{in}) and outer surface (CSA_{out}), and the corresponding

circumferences (C_{muc}, C_{in} and C_{out}).

For each of the three sur- and interfaces the *circumferential stretch ratio* (a deformation measure) was computed as the relative elongation of each circumference: $\lambda_\theta = c/c_{\text{zero-pressure}}$, where $c_{\text{zero-pressure}}$ denotes the circumference of the unloaded segment. The $c_{\text{zero-pressure}}$ was measured directly from ultrasound images at the start of the butylscopolamine distension. The *circumferential strain* at each circumference (mucosa, submucosa-muscle and outer, respectively) was represented by the Green strain:

$$\varepsilon_\theta = \frac{c^2 - c_{\text{zero-pressure}}^2}{2c_{\text{zero-pressure}}^2} \quad (1)$$

The radial deformation of the mucosal and muscle layers were computed as *radial stretch ratio*: $\lambda_r = h/h_{\text{zero-pressure}}$, where h and $h_{\text{zero-pressure}}$ represents the thickness of the distended and butylscopolamine relaxed layers.

Assuming a circular shape, the wall thicknesses were calculated as:

$$h_{\text{total}} = r_{\text{out}} - r_{\text{muc}} = \sqrt{\text{CSA}_{\text{out}}/\pi} - \sqrt{\text{CSA}_{\text{muc}}/\pi}, \quad (2a)$$

$$h_{\text{muc}} = r_{\text{in}} - r_{\text{muc}} = \sqrt{\text{CSA}_{\text{in}}/\pi} - \sqrt{\text{CSA}_{\text{muc}}/\pi}, \text{ and} \quad (2b)$$

$$h_{\text{muscle}} = r_{\text{out}} - r_{\text{in}} = \sqrt{\text{CSA}_{\text{out}}/\pi} - \sqrt{\text{CSA}_{\text{in}}/\pi}. \quad (2c)$$

The *longitudinal stretch ratio* of each layer which indicate the degree of longitudinal deformation was calculated as $\lambda_z = \frac{1}{\lambda_\theta \lambda_r}$ assuming incompressibility ($\lambda_\theta \lambda_r \lambda_z = 1$) of the tissue^[35].

Since the esophagus is thick-walled the pressure and stress decay through the wall is expected to be non-linear^[50]. Hence, the distribution of the circumferential stress through the wall was computed as:

$$\tau_{\theta,r} = \frac{r_{\text{out}}^2 r_{\text{muc}}^2 \Delta p}{r^2 (r_{\text{out}}^2 - r_{\text{muc}}^2)} + \frac{\Delta p r_{\text{muc}}^2}{r_{\text{out}}^2 - r_{\text{muc}}^2} \quad (3)$$

where r denotes the radial location inside the wall^[30,50]. The radii r_{out} and r_{muc} was calculated as: $r_{\text{out}} = \sqrt{\text{CSA}_{\text{out}}/\pi}$ and $r_{\text{muc}} = \sqrt{\text{CSA}_{\text{muc}}/\pi}$. Δp represents the bag pressure corrected for the baseline pressure (representing the mediastinal resting pressure) before the butylscopolamine distensions.

To obtain mechanical constants of each layer the circumferential stress-strain relationship of the mucosal surface, the interface between submucosa and muscle layer, and the outer surface layers for each subject were plotted. α and β constants were obtained using non-linear curve fitting (Microcal Origin 6.0, Microcal Software Inc., Northampton, MA, USA) approximating the equation^[51]:

$$\tau_\theta = \beta(e^{\alpha \varepsilon_\theta} - 1) \quad (4)$$

The computed stiffness parameter is dependent on passive stretch and the contribution from active muscle contraction or tone. Administration of butylscopolamine abolishes smooth muscle contractions whereby the passive properties can be assessed.

Analysis of duodenal distensions

The duodenal ultrasound images were captured as

described at the start of each distension and between the distension-induced contractions (see above). This allowed computation of the luminal duodenal *CSA* and mucosal circumference *c*. The *circumferential stretch ratio* was computed as the relative elongation of the mucosal surface (circumference *c*) during distension:

$$\lambda_\theta = c / c_{\text{zero-pressure}} \quad (5)$$

Since the ultrasound imaging not always allowed measurements at the start of the distension (artifacts due to air), $c_{\text{zero-pressure}}$ was in some cases approximated by a double logarithmic fitting of the circumference-pressure data. The validity of the fitting procedure was verified from the experiments with good imaging quality. A good agreement between fitted and measured values was found^[51]. The *circumferential strain* was computed as the Green strain, see Eq. 1.

As an approximation the wall thickness was not taken into account^[35] and consequently the *circumferential tension* was computed using Laplace's law:

$$T_\theta = \Delta p r \quad (7)$$

where *r* denotes the inner duodenal radius assuming circular shape: $r = \sqrt{CSA/\pi}$. The wall tension is the integration of the stresses through the wall (the stress moment).

The circumferential tension-strain relationships for each subject were plotted to obtain the curve fitting constants. The α and β constants were obtained using non-linear curve fitting (Microcal Origin 6.0, Microcal Software Inc., Northampton, MA, USA) using a modification of Fung's approach^[51]:

$$T_\theta = \beta(e^{\alpha\lambda_\theta} - 1) \quad (8)$$

Limitation of the ultrasound technique

The ultrasound technique suffers from limitations too. At low degrees of distension convolutions of the plastic bag resulted in artifacts (air and plastic folds) and in many cases only the bag-mucosa, submucosa-muscle and muscle-adventitia interfaces could be clearly identified. At low degrees of distension it was difficult to clearly identify the inner circumference of the duodenum. This was due to small amounts of air outside the bag and folds in the bag resulting in artifacts and the fact that the bag might not always be in contact with the wall in the entire circumference at low degrees of distension. To compensate for this problem, the mucosal circumference at low degrees of distension was approximated using double-logarithmic curve fitting. Other limitations were that the ultrasound system used in this study did not provide sufficient image quality for accurate measurement of the wall thickness in the entire duodenal circumference, especially at high degrees of distension.

REFERENCES

- Rundles RW. Diabetic Neuropathy. *Medicine* 1945; **24**: 111-160
- Ko GT, Chan WB, Chan JC, Tsang LW, Cockram CS. Gastrointestinal symptoms in Chinese patients with Type 2 diabetes mellitus. *Diabet Med* 1999; **16**: 670-674
- Folwaczny C, Riepl R, Tschöp M, Landgraf R. Gastrointestinal involvement in patients with diabetes mellitus: Part I (first of two parts). Epidemiology, pathophysiology, clinical findings. *Z Gastroenterol* 1999; **37**: 803-815
- Spångéus A, El-Salhy M, Suhr O, Eriksson J, Lithner F. Prevalence of gastrointestinal symptoms in young and middle-aged diabetic patients. *Scand J Gastroenterol* 1999; **34**: 1196-1202
- Horowitz M, Samsom M. Gastrointestinal Function in Diabetes Mellitus. Chichester: John Wiley & Sons Ltd., 2004: 1-27
- Mearin F, Malagelada JR. Gastroparesis and dyspepsia in patients with diabetes mellitus. *Eur J Gastroenterol Hepatol* 1995; **7**: 717-723
- Horowitz M, Edelbroek M, Fraser R, Maddox A, Wishart J. Disordered gastric motor function in diabetes mellitus. Recent insights into prevalence, pathophysiology, clinical relevance, and treatment. *Scand J Gastroenterol* 1991; **26**: 673-684
- Britland ST, Young RJ, Sharma AK, Lee D, Ah-See AK, Clarke BF. Vagus nerve morphology in diabetic gastropathy. *Diabet Med* 1990; **7**: 780-787
- Holtmann G, Goebell H, Talley NJ. Gastrointestinal sensory function in functional dyspepsia. *Gastroenterology* 1995; **109**: 331-332
- Samsom M, Salet GA, Roelofs JM, Akkermans LM, Vanberge-Henegouwen GP, Smout AJ. Compliance of the proximal stomach and dyspeptic symptoms in patients with type I diabetes mellitus. *Dig Dis Sci* 1995; **40**: 2037-2042
- Rayner CK, Samsom M, Jones KL, Horowitz M. Relationships of upper gastrointestinal motor and sensory function with glycemic control. *Diabetes Care* 2001; **24**: 371-381
- Hebbard GS, Samsom M, Sun WM, Dent J, Horowitz M. Hyperglycemia affects proximal gastric motor and sensory function during small intestinal triglyceride infusion. *Am J Physiol* 1996; **271**: G814-G819
- Clouse RE, Lustman PJ, Reidel WL. Correlation of esophageal motility abnormalities with neuropsychiatric status in diabetics. *Gastroenterology* 1986; **90**: 1146-1154
- Nowak TV, Harrington B, Weisbruch JP, Kalbfleisch JH. Structural and functional characteristics of muscle from diabetic rodent small intestine. *Am J Physiol* 1990; **258**: G690-G698
- Ulrich P, Cerami A. Protein glycation, diabetes, and aging. *Recent Prog Horm Res* 2001; **56**: 1-21
- Sánchez SS, Genta SB, Aybar MJ, Honoré SM, Villecco EI, Sánchez Riera AN. Changes in the expression of small intestine extracellular matrix proteins in streptozotocin-induced diabetic rats. *Cell Biol Int* 2000; **24**: 881-888
- Zoubi SA, Mayhew TM, Sparrow RA. The small intestine in experimental diabetes: cellular adaptation in crypts and villi at different longitudinal sites. *Virchows Arch* 1995; **426**: 501-507
- Zoubi SA, Williams MD, Mayhew TM, Sparrow RA. Number and ultrastructure of epithelial cells in crypts and villi along the streptozotocin-diabetic small intestine: a quantitative study on the effects of insulin and aldose reductase inhibition. *Virchows Arch* 1995; **427**: 187-193
- Jørgensen CS, Ahrensberg JM, Gregersen H, Flyvbjerg A. Tension-strain relations and morphometry of rat small intestine in experimental diabetes. *Dig Dis Sci* 2001; **46**: 960-967
- Zhao J, Yang J, Gregersen H. Biomechanical and morphometric intestinal remodelling during experimental diabetes in rats. *Diabetologia* 2003; **46**: 1688-1697
- Zhao J, Liao D, Yang J, Gregersen H. Viscoelastic behavior of small intestine in streptozotocin-induced diabetic rats. *Dig Dis Sci* 2003; **48**: 2271-2277
- Yang J, Zhao J, Zeng Y, Gregersen H. Biomechanical properties of the rat oesophagus in experimental type-1 diabetes. *Neurogastroenterol Motil* 2004; **16**: 195-203
- Hollis JB, Castell DO, Braddom RL. Esophageal function in diabetes mellitus and its relation to peripheral neuropathy. *Gastroenterology* 1977; **73**: 1098-1102
- Loo FD, Dodds WJ, Soergel KH, Arndorfer RC, Helm JF, Hogan WJ. Multipeaked esophageal peristaltic pressure waves

- in patients with diabetic neuropathy. *Gastroenterology* 1985; **88**: 485-491
- 25 **Assentoft JE**, Gregersen H, O'Brien WD. Determination of biomechanical properties in guinea pig esophagus by means of high frequency ultrasound and impedance planimetry. *Dig Dis Sci* 2000; **45**: 1260-1266
 - 26 **Jørgensen CS**, Dall FH, Jensen SL, Gregersen H. A new combined high-frequency ultrasound-impedance planimetry measuring system for the quantification of organ wall biomechanics *in vivo*. *J Biomech* 1995; **28**: 863-867
 - 27 **Takeda T**, Kassab G, Liu J, Puckett JL, Mittal RR, Mittal RK. A novel ultrasound technique to study the biomechanics of the human esophagus *in vivo*. *Am J Physiol Gastrointest Liver Physiol* 2002; **282**: G785-G793
 - 28 **Takeda T**, Kassab G, Liu J, Nabae T, Mittal RK. Effect of atropine on the biomechanical properties of the oesophageal wall in humans. *J Physiol* 2003; **547**: 621-628
 - 29 **Takeda T**, Nabae T, Kassab G, Liu J, Mittal RK. Oesophageal wall stretch: the stimulus for distension induced oesophageal sensation. *Neurogastroenterol Motil* 2004; **16**: 721-728
 - 30 **Frøkjær JB**, Andersen SD, Lundbye-Christensen S, Funch-Jensen P, Drewes AM, Gregersen H. Sensation and distribution of stress and deformation in the human oesophagus. *Neurogastroenterol Motil* 2006; **18**: 104-114
 - 31 **Frøkjær JB**, Andersen SD, Drewes AM, Gregersen H. Ultrasound-determined geometric and biomechanical properties of the human duodenum. *Dig Dis Sci* 2006; **51**: 1662-1669
 - 32 **Gao C**, Arendt-Nielsen L, Liu W, Petersen P, Drewes AM, Gregersen H. Sensory and biomechanical responses to ramp-controlled distension of the human duodenum. *Am J Physiol Gastrointest Liver Physiol* 2003; **284**: G461-G471
 - 33 **Frøkjær JB**, Andersen SD, Gale J, Arendt-Nielsen L, Gregersen H, Drewes AM. An experimental study of viscerovisceral hyperalgesia using an ultrasound-based multimodal sensory testing approach. *Pain* 2005; **119**: 191-200
 - 34 **Drewes AM**, Pedersen J, Liu W, Arendt-Nielsen L, Gregersen H. Controlled mechanical distension of the human oesophagus: sensory and biomechanical findings. *Scand J Gastroenterol* 2003; **38**: 27-35
 - 35 **Gregersen H**. Biomechanics of the Gastrointestinal Tract. London: Springer-Verlag, 2003: 1-262
 - 36 **Björnsson ES**, Urbanavicius V, Eliasson B, Attvall S, Smith U, Abrahamsson H. Effects of hyperglycemia on interdigestive gastrointestinal motility in humans. *Scand J Gastroenterol* 1994; **29**: 1096-1104
 - 37 **Rayner CK**, Smout AJ, Sun WM, Russo A, Semmler J, Sattawatthamrong Y, Tellis N, Horowitz M. Effects of hyperglycemia on cortical response to esophageal distension in normal subjects. *Dig Dis Sci* 1999; **44**: 279-285
 - 38 **Gregersen H**, Christensen J. Gastrointestinal tone. *Neurogastroenterol Motil* 2000; **12**: 501-508
 - 39 **Frøkjær JB**, Andersen SD, Ejskaer N, Funch-Jensen P, Arendt-Nielsen L, Gregersen H, Drewes AM. Gut sensations in diabetic autonomic neuropathy. *Pain* 2007; (Epub ahead of print)
 - 40 **Silber W**. Diabetes and oesophageal dysfunction. *Br Med J* 1969; **3**: 688-690
 - 41 **Russell CO**, Gannan R, Coatsworth J, Neilsen R, Allen F, Hill LD, Pope CE. Relationship among esophageal dysfunction, diabetic gastroenteropathy, and peripheral neuropathy. *Dig Dis Sci* 1983; **28**: 289-293
 - 42 **Keshavarzian A**, Iber FL, Nasrallah S. Radionuclide esophageal emptying and manometric studies in diabetes mellitus. *Am J Gastroenterol* 1987; **82**: 625-631
 - 43 **Kinekawa F**, Kubo F, Matsuda K, Fujita Y, Tomita T, Uchida Y, Nishioka M. Relationship between esophageal dysfunction and neuropathy in diabetic patients. *Am J Gastroenterol* 2001; **96**: 2026-2032
 - 44 **Drewes AM**, Pedersen J, Reddy H, Rasmussen K, Funch-Jensen P, Arendt-Nielsen L, Gregersen H. Central sensitization in patients with non-cardiac chest pain: a clinical experimental study. *Scand J Gastroenterol* 2006; **41**: 640-649
 - 45 **Pedersen J**, Gao C, Egekvist H, Bjerring P, Arendt-Nielsen L, Gregersen H, Drewes AM. Pain and biomechanical responses to distention of the duodenum in patients with systemic sclerosis. *Gastroenterology* 2003; **124**: 1230-1239
 - 46 **Jermendy G**, Fornet B, Koltai MZ, Pogátsa G. Correlation between oesophageal dysmotility and cardiovascular autonomic dysfunction in diabetic patients without gastrointestinal symptoms of autonomic neuropathy. *Diabetes Res* 1991; **16**: 193-197
 - 47 **Tahara T**, Yamamoto T. Morphological changes of the villous microvascular architecture and intestinal growth in rats with streptozotocin-induced diabetes. *Virchows Arch A Pathol Anat Histopathol* 1988; **413**: 151-158
 - 48 **Ejskjaer NT**, Bradley JL, Buxton-Thomas MS, Edmonds ME, Howard ER, Purewal T, Thomas PK, Watkins PJ. Novel surgical treatment and gastric pathology in diabetic gastroparesis. *Diabet Med* 1999; **16**: 488-495
 - 49 **Reddy GK**. AGE-related cross-linking of collagen is associated with aortic wall matrix stiffness in the pathogenesis of drug-induced diabetes in rats. *Microvasc Res* 2004; **68**: 132-142
 - 50 **Fung YC**. A First Course in Continuum Mechanics. Englewood Cliffs, NJ: Prentice Hall, 1994: 1-412
 - 51 **Fung YC**. Biomechanics, Motion, Flow and Growth. New York: Springer Verlag, 1990: 499-546

S- Editor Liu Y L- Editor Negro F E- Editor Ma WH

Ferucarbotran *versus* Gd-DTPA-enhanced MR imaging in the detection of focal hepatic lesions

Wei-Zhong Cheng, Meng-Su Zeng, Fu-Hua Yan, Shen-Xiang Rao, Ji-Zhang Shen, Cai-Zhong Chen, Shu-Jie Zhang, Wei-Bin Shi

Wei-Zhong Cheng, Meng-Su Zeng, Fu-Hua Yan, Shen-Xiang Rao, Ji-Zhang Shen, Cai-Zhong Chen, Shu-Jie Zhang, Wei-Bin Shi, Department of Radiology, Zhongshan Hospital, Fudan University and Department of Medical Imaging, Shanghai Medical School, Fudan University, Shanghai 200032, China
Correspondence to: Meng-Su Zeng, Department of Radiology, Zhongshan Hospital, 180 Fenglin Rd, Shanghai 200032, China. zms@zshospital.net
Telephone: +86-21-64041990-2130 Fax: +86-21-64037258
Received: March 27, 2007 Revised: May 12, 2007

Abstract

AIM: To evaluate the efficacy of ferucarbotran-enhanced MR imaging in the detection of focal hepatic lesions compared to plain and Gd-DTPA-enhanced MR imaging.

METHODS: Fifty-nine patients with suspected focal hepatic lesions were admitted to the study. Plain MR imaging (FSE T₂WI with fat suppression and GRE T₁WI sequences) and Gd-DTPA dynamic enhanced MR of the liver were initially performed followed by ferucarbotran-enhanced MR imaging 48 h later (including GRE T₁WI, FSE T₂WI with fat suppression, and GRE T₂*WI sequences). Images were reviewed independently by three observers. Results were correlated with surgery and pathologic examination or reference examination, and sensitivity was statistically calculated for the different MR imaging sequences.

RESULTS: Among all confirmed lesions ($n = 133$), ferucarbotran-enhanced MR imaging revealed 130 lesions on FSE T₂WI with fat suppression, 115 lesions on dynamic T₁WI GRE, and 127 lesions on GRE T₂*WI. Pre-contrast MR imaging revealed only 84 lesions on GRE T₁WI and 106 lesions on FSE T₂WI with fat suppression, while Gd-DTPA dynamic enhanced GRE T₁WI revealed 123 lesions. For 44 micro-lesions (< 1.0 cm) in all patients the detection rates were as follows: ferucarbotran-enhanced FSE T₂WI with fat suppression, 93.2% (41/44); ferucarbotran-enhanced GRE T₂*WI, 88.6% (39/44); Gd-DTPA dynamic-enhanced GRE T₁WI, 79.5% (35/44); pre-contrast FSE T₂WI with fat suppression, 54.5% (24/44); and pre-contrast GRE T₁WI, 34.1% (15/44). In detecting micro-lesions, statistically significant difference was found for Ferucarbotran-enhanced FSE T₂WI with fat suppression and GRE T₂*WI sequences compared to the other sequences ($P < 0.05$).

CONCLUSION: Ferucarbotran-enhanced FSE T₂WI with fat suppression and GRE T₂*WI sequences are superior in detecting micro-lesions (< 1 cm) in comparison with plain and Gd-DTPA dynamic-enhanced MR imaging.

© 2007 WJG. All rights reserved.

Key words: Liver disease; Contrast media; Superparamagnetic iron oxide; Magnetic resonance imaging

Cheng WZ, Zeng MS, Yan FH, Rao SX, Shen JZ, Chen CZ, Zhang SJ, Shi WB. Ferucarbotran *versus* Gd-DTPA-enhanced MR imaging in the detection of focal hepatic lesions. *World J Gastroenterol* 2007; 13(36): 4891-4896

<http://www.wjgnet.com/1007-9327/13/4891.asp>

INTRODUCTION

The detection of focal liver lesions in patients with liver cancer is a very important challenge because failure to detect cancerous lesions can have major clinical consequences. High accuracy in liver cancer detection can improve the efficacy of treatment, including partial hepatectomy, liver transplantation, radiofrequency ablation, percutaneous ethanol injection, transarterial chemoembolization, or (commonly) a combination of these methods^[1-5]. Currently, magnetic resonance (MR) is increasingly used in the detection of hepatic lesions^[6]. With the use of conventional extracellular contrast agents such as gadolinium chelates, analysis of enhancement patterns on T₁-weighted dynamic imaging during the different vascular phases is an important tool in the detection and characterization of focal hepatic lesions. Post-contrast imaging has been shown to be superior to conventional plain MR imaging in detecting hepatic lesions^[7]. It is known, however, that 40%-60% of cancer nodules, especially those in cirrhotic liver that are smaller than 10 mm, are missed at ultrasonography (US), computed tomography (CT)^[8], and MR^[9]. Given this background, a possible solution to this problem is the use of liver-specific MR contrast materials: that is, agents that are targeted to either the hepatocytes or the Kupffer cells. Ferucarbotran is available as a new superparamagnetic iron oxide (SPIO) agent for liver imaging in most European countries and some Asian countries^[10,11]. The present study was designed

as an open-label, within-patient comparison of the diagnostic performance of non-enhanced, gadolinium-enhanced, and ferucarbotran-enhanced MR imaging, in terms of lesion detection and characterization according to the Phase III clinical trial of ferucarbotran in China. This article is mainly concerned with evaluating efficacy in the detection of hepatic lesions.

MATERIALS AND METHODS

Patients

The study protocol was approved by the Zhongshan Hospital Ethics Committee. A total of 59 patients (40 men, 19 women; mean age, 48.9 year; age range, 26-68 year) with at least one suspected focal hepatic lesion were enrolled in the study and received test contrast material between December 2003 and July 2004. Each patient gave written or witnessed informed consent and was evaluated for eligibility. Each patient underwent a reference-standard examination. The best method to confirm imaging findings—that is, pathologic confirmation—was unavailable for every patient in this study.

Contrast material

Ferucarbotran (Resovist, Schering, Berlin, Germany) was preloaded into a 2.25 mL connecting intravenous tube (Connection Tubing; Clinico, Bad Hersfeld, Germany) and manually injected as a bolus through a filter with 5- μ m pore size; the connecting catheter was flushed with 10 mL of saline solution within 3 s of injection (injection rate, approximately 2-3 mL/s). Patients with a body weight of 60 kg or more received 1.4 mL of ferucarbotran, while those with a body weight of less than 60 kg received 0.9 mL (range, 7.0-12.9 μ mol iron/kg); 1 mL ferucarbotran contains 28 mg of iron. Gd-DTPA (Magnevist, Schering, Erlangen, Germany) was manually administered as an intravenous bolus injection at a dose of 0.2 mL/kg (corresponding to 0.1 mmol/kg) with a flow rate of 2-3 mL/s.

MR imaging protocol

MR imaging was performed with a superconducting magnet operating at 1.5T (Signa, GE Medical Systems, Milwaukee, USA). The imaging protocol consisted of pre- and post-contrast imaging at 25, 60, 180, and 480 s after the administration of each contrast material, with an interval of 48 h between the injection of each. Every examination consisted of T2-weighted fast spin-echo (FSE) with fat suppression, T1-weighted fast multiplanar spoiled gradient-recalled (SPGR), and T2* GRE sequences (Table 1). The imaging factors were the same for all patients, and all sequences were performed before and after Gd-DTPA contrast agent and ferucarbotran administration.

Imaging assessment

According to the surgical and pathologic confirmation or reference examination, all images from the MR imaging sequences were interpreted independently by three radiologists; the number of lesions was then calculated, excluding cysts. Consensus reading was necessary to ensure

that both observers assessed the same lesion. Lesions were divided into three groups according to lesion size (< 1 cm, 1-3 cm, > 3 cm).

Statistical analysis

The detection rates for the different sequences (FSE T2WI with fat suppression, GRE T1WI, and GRE T2*WI) were compared using Pearson's chi-square test and the Fisher exact probability test. $P < 0.05$ was considered to indicate statistical significance.

RESULTS

According to pathologic confirmation and the reference examinations, a total of 133 lesions were present among the subjects (85 lesions were confirmed by pathology; 75 hepatocellular carcinoma lesions, 35 metastatic lesions, 7 dysplastic nodules, 6 hemangiomas, 5 cases of focal nodular hyperplasia, 2 tuberculomas, 1 inflammatory pseudotumor, 2 angioleiomyolipomas). Twenty-one lesions were larger than 3.0 cm, 68 were 1-3 cm, and 44 were smaller than 1 cm. On ferucarbotran-enhanced MR imaging, FSE T2WI with fat suppression revealed 130 lesions, dynamic T1WI GRE revealed 115 lesions, and GRE T2*WI revealed 127 lesions (Figure 1 A-H). On pre-contrast MR imaging, GRE T1WI revealed only 84 lesions and FSE T2WI with fat suppression revealed 106 lesions. Gd-DTPA dynamic-enhanced GRE T1WI revealed 123 lesions (Table 2). Three lesions were not found by any observer on any sequence: All were smaller than 0.8 mm in diameter and were metastases from mammary adenocarcinoma and colorectal cancer. In another patient, a 0.3 mm-diameter subcapsular metastasis was detected only by ferucarbotran-enhanced FSE (Figure 2A-F).

Table 2 shows the detectability among 44 micro-lesions (< 1.0 cm) for all patients as follows: ferucarbotran-enhanced FSE T2WI with fat suppression, 93.2% (41/44); ferucarbotran-enhanced GRE T2*WI, 88.6% (39/44); Gd-DTPA dynamic-enhanced GRE T1WI, 79.5% (35/44); pre-contrast FSE T2WI with fat suppression, 54.5% (24/44); and pre-contrast GRE T1WI, 34.1% (15/44). The detectability of ferucarbotran-enhanced FSE T2WI with fat suppression and ferucarbotran-enhanced GRE T2*WI sequences was significantly greater than that of the other sequences ($P < 0.01$).

DISCUSSION

Ferucarbotran contains superparamagnetic iron oxide nanoparticles (maghemite [γ -Fe₂O₃] and magnetite [γ -Fe₃O₄]) coated with a carboxydextran shell, and was developed as a new intravenous liver-specific contrast agent. Its T1 relaxivity is 25.4 mmol/L per second, with T2 relaxivity of 151.0 mmol/L. second. The blood half-life is similar to that of ferumoxides (another SPIO agent), but the mean particle size is smaller (60 nm)^[10,11]. Ferucarbotran is taken up by reticuloendothelial system (RES) cells in the liver, spleen, bone marrow, and lymph nodes. Hydrolytic enzymes degrade intracellular SPIO particles causing a loss of R2 relaxivity as the iron loses its

Table 1 MR sequences and parameters

Sequence	TR	TE	NEX	Matrix	FOV (cm)	Thickness	Intersection gap (mm)	Flip angle (mm)
T2-FSE/TSE with fat suppression	3333-4000	98	2	256 × 160	36 × 36	8	2	-
GRE T1WI	150	2	1	256 × 128	36 × 36	8	2	90°
GRE T2*WI	150	11.5	1	256 × 160	36 × 36	8	2	10°

Table 2 Comparative analysis of lesion detection for 133 lesions in 59 patients using pre-contrast, ferucarbotran-enhanced, and Gd-DTPA-enhanced sequences

Sequence	n (%)		
	< 1 cm (n = 44)	1-3 cm (n = 68)	> 3 cm (n = 21)
1 Pre-contrast T1WI GRE	15 (34.1) ^b	48 (70.6) ^b	21 (100.0)
2 Pre-contrast FSE T2WI with fat suppression	24 (54.5) ^b	61 (89.7)	21 (100.0)
3 Dynamic Gd-DTPA-enhanced T1WI GRE	35 (79.5) ^a	67 (98.5)	21 (100.0)
4 Dynamic ferucarbotran-enhanced T1WI GRE	32 (72.7)	62 (91.2)	21 (100.0)
5 Delayed ferucarbotran-enhanced FSE T2WI with fat suppression	41 (93.2)	68 (100)	21 (100.0)
6 Delayed ferucarbotran-enhanced GRE T2*WI	39 (88.6)	67 (98.5)	21 (100.0)

^a $P < 0.05$, ^b $P < 0.01$, *vs* (5), (6).

crystalline structure^[12]. Within minutes of administration, 80% of the injected dose of SPIO agent efficiently accumulates in the liver, while approximately 5%-10% of the injected dose accumulates in the spleen^[13-15]. On T2-weighted sequences there is a marked decrease in the signal intensity of normal liver and spleen; because malignant tumor tissue typically lacks a substantial number of Kupffer cells or has a lower activity of phagocytic cells, it appears as hyperintense/bright lesions that are contrasted against the hypointense/black liver^[11,16].

In the detection of micro-lesions (< 1.0 cm) in the present study, delayed T2-weighted ferucarbotran-enhanced FSE with fat suppression and T2*-weighted ferucarbotran-enhanced GRE provided higher sensitivity in MR imaging than pre-contrast (T1-weighted GRE) images (93.2% and 88.6%, respectively, *vs* 34.1%). More lesions were detected on T2-weighted ferucarbotran-enhanced FSE than on dynamic Gd-DTPA-enhanced images in 16%-20% of our patients. It had statistically significant difference in detecting micro-lesions ($P = 0.021$), which is in agreement with previous studies^[11,16-18]. In the present study, even a 0.3 mm-diameter metastasis was detected by T2-weighted ferucarbotran-enhanced FSE with fat suppression. The loss of liver parenchyma signal intensity after ferucarbotran administration improves lesion-to-liver contrast, which in turn improves detection (especially in micro-lesions), visualization, delineation, and overall diagnostic confidence^[19] (Figure 3A-D). Although diagnostic confidence is a subjective parameter, it is relevant in the clinical situation because a radiologist must determine the confidence with which findings observed on images are correct. Evaluation of diagnostic certainty was also recommended by Thornbury in his six-tier model

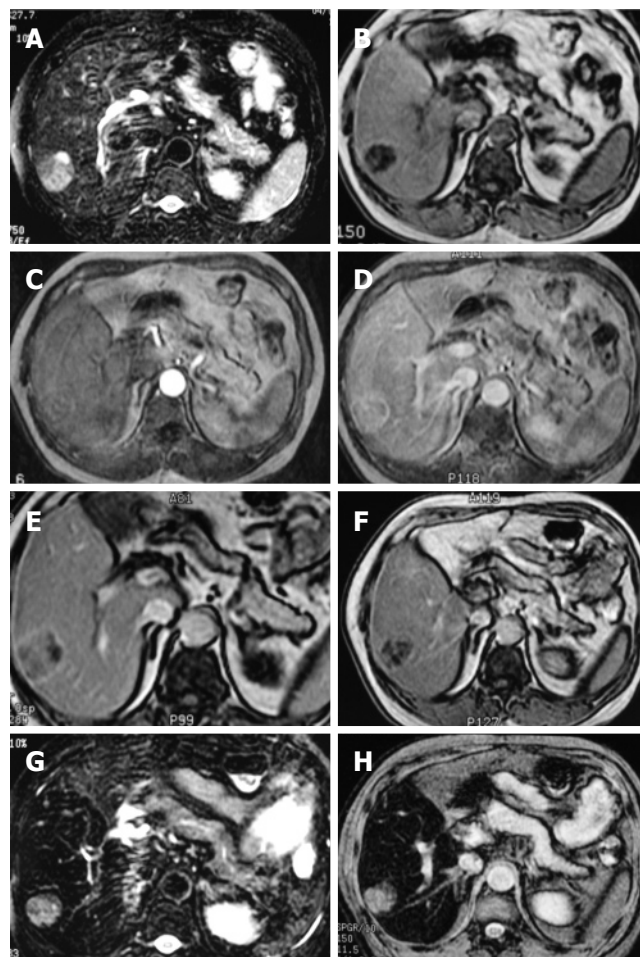


Figure 1 Images of HCC. **A:** The lesion appears hyperintense on the pre-contrast T2WI FSE image; **B:** The lesion appears hypointense on the pre-contrast T1WI GRE image; **C:** The lesion enhances inhomogeneously during the hepatic arterial phase; **D:** Ring-enhancement is observed during the portal venous phase; **E, F:** Dynamic T1WI GRE images obtained after administration of ferucarbotran show ring-enhancement of an asymmetric moderately hyperintense lesion; **G, H:** A markedly hyperintense lesion with surrounding hypointense liver parenchyma is shown during the accumulation phase on ferucarbotran-enhanced T2WI and T2*WI images.

of efficacy^[20]. Diagnostic confidence conceptualizes the diagnostic thinking efficacy (one tier of the Thornbury model) and links the technical and diagnostic efficacy of a contrast agent to the therapy of the patient. Higher accuracy in liver cancer detection potentially changes the clinical treatment of patients with liver cancer. The report of Ros *et al* states that the proportionate change in clinical treatment was as high as 59% after examination using SPIO-enhanced MR^[21]. For detection of lesions larger than 1 cm, there was no significant difference among the findings of the different sequences (excluding pre-

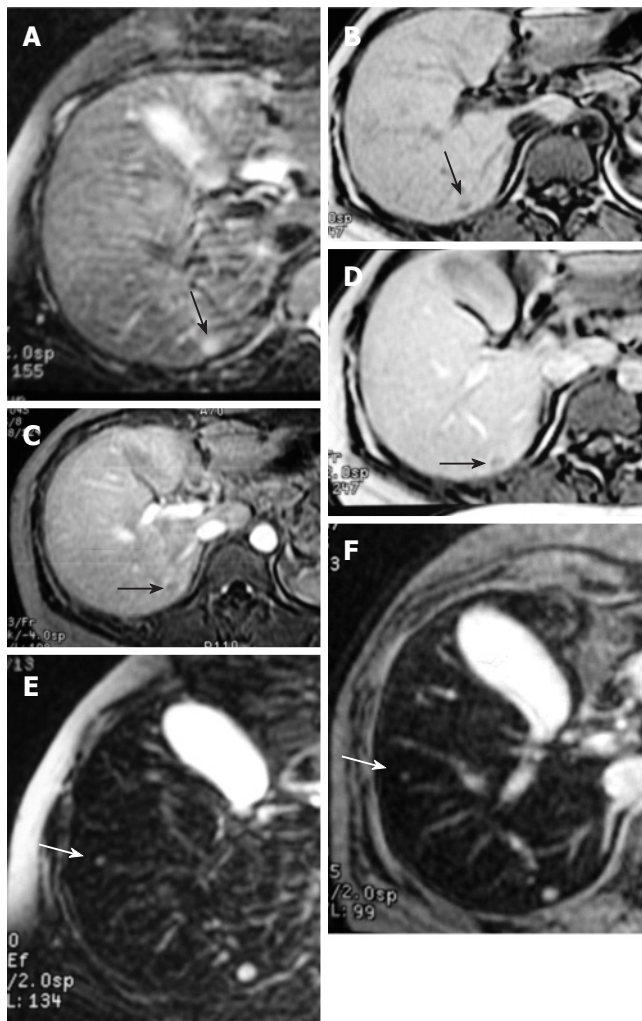


Figure 2 Liver metastasis from mammary adenocarcinoma. **A, B:** Only one lesion (black arrow) was found on the pre-contrast T2WI and T1WI GRE images; **C, D:** Only one lesion (black arrow) was found on dynamic T1WI GRE images obtained after administration of Gd-DTPA; **E, F:** An additional small metastasis (0.3 mm, white arrow) was detected on T2WI and T2*WI images during the accumulation phase.

contrast sequences); however, diagnostic confidence was determined for the same reason as that described above.

Ferucarbotran is an SPIO agent that can be injected as a bolus^[10]. It contains particles with smaller mean hydrodynamic diameter than that of the stock solution, which has a stronger T1-effect and longer blood half-life because of slower uptake into the RES. Previous studies using animals found that iron particles begin attaching to the Kupffer cells 3 min after administration^[22]. The R1 relaxivity of ferucarbotran varies considerably within the range of diagnostically applied proton Larmor frequency (i.e., field strength); however, R1 relaxivity at 40 MHz with 12.3 mmol/L per second is still four times higher than the R1 relaxivity of low molecular gadolinium chelates. The very high R2/R1 ratio is characteristic of superparamagnetic colloids of the SPIO type. This ratio varies from 6 to 15 as the Larmor frequency increases from 10 to 40 MHz, a fact which might be in favor of the use of lower imaging fields to better utilize the T1 effect of these materials^[12]. In fact, this subfraction contributes

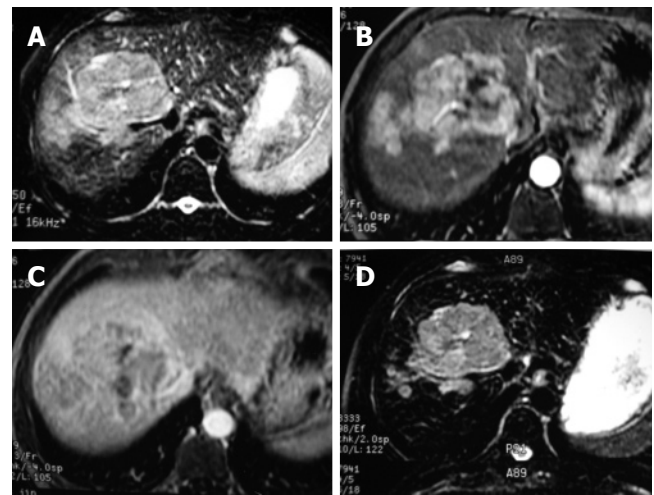


Figure 3 HCC image. **A:** The lesion appears hyperintense on the pre-contrast T2WI FSE image, but is not clearly delineated; **B:** The lesion is markedly enhanced during the hepatic arterial phase; **C:** The lesion appears hypointense during the portal venous phase, but is not clearly delineated; **D:** The main lesion and satellite nodules are clearly delineated from the surrounding hypointense liver parenchyma on the ferucarbotran-enhanced T2WI image during the accumulation phase.

to the signal change on T1-weighted images and is the reason why the signal characteristics are comparable to those of angiographic agents with a blood-pool effect. This subfraction also explains the biexponential blood half-life of ferucarbotran, which has also been tested for its capability in contrast-enhanced MRA^[23]. Enhancement increased with the degree of T1-weighting and shifted towards higher concentrations with shorter echo times. The T1 effect of ferucarbotran is a function of dose and concentration in plasma, and can be monitored during a time window in which the plasma concentration stays below a certain level depending on pulse sequence parameters. Nevertheless, in the present study, dynamic ferucarbotran-enhanced T1-weighted GRE/FLASH showed no superiority over dynamic Gd-DTPA-enhanced T1-weighted GRE/FLASH in the detection of lesions. The benefit of dynamic T1-weighted MR imaging techniques is in the characterization of lesions; however, in many respects dynamic imaging with ferucarbotran is different from that with Gd-DTPA. In dynamic T1-weighted images obtained with ferucarbotran, the signal increase is observed to be lower but longer lasting, as described by Amelie *et al*^[24].

Ferucarbotran has an advantage over ferumoxides (Feridex, Advanced Magnetix; Guerbet), which is the only marketed SPIO in most countries. Although it is well known that ferumoxides have relatively high R1 values (up to four or five times higher than those of gadolinium-based contrast agents)^[10,25,26], these T1 effects cannot be readily exploited for dynamic T1-weighted MR imaging. Bolus administration of ferumoxides has not been recommended because of the possible side effects (lumbar pain and cardiovascular problems, including dose-dependent hypotensive reactions) that positively correlate to the rate of injection; therefore, this compound must be administered as a slow drip infusion^[13]. Ferucarbotran used in this study has few side effects^[10,11] and can be given as a

rapid bolus injection. No adverse reactions were observed in any of the patients in the present study; however, as for any SPIO contrast agent, the efficacy of ferucarbotran depends on the number of Kupffer cells or their activity. For example, some well-differentiated hepatocellular carcinoma lesions still contain a certain amount of Kupffer cells and which could confuse us to make the correct diagnosis^[27,28].

In conclusion, ferucarbotran is a safe contrast material for MR imaging of local liver lesions. The use of ferucarbotran-enhanced images improves diagnostic confidence and lesion detection, especially in the detection of micro lesions (< 1 cm). Ferucarbotran can be rapidly injected without clinically important adverse events, enabling dynamic MR imaging to be performed. As a dual-function and complementary MR imaging agent, ferucarbotran-enhanced images in combination with dynamic Gd-DTPA-enhanced images are helpful in improving the accuracy of differential diagnoses for focal hepatic lesions.

COMMENTS

Background

In patients with liver cancer, the detection of focal liver lesions is one of the most important challenges. High accuracy in liver cancer detection can improve the efficacy of treatment. It is known, however, that 40%-60% of cancer nodules, especially those that are smaller than 10 mm in cirrhotic liver, are missed on ultrasonography (US), computed tomography (CT) and magnetic resonance (MR).

Research frontiers

Ferucarbotran, a liver-specific MR contrast material targeted to the Kupffer cells, is available as a new superparamagnetic iron oxide (SPIO) agent for liver imaging. A current research hotspot involves the evaluation of two contrast media for their efficacy in detecting hepatic lesions.

Innovations and breakthroughs

Ferucarbotran is a safe contrast material for MR imaging of focal liver lesions. The use of ferucarbotran-enhanced images improves diagnostic confidence and lesion detection, especially in the detection of micro lesions.

Applications

Ferucarbotran-enhanced images combined with dynamic Gd-DTPA-enhanced images aid in improving the accuracy of differential diagnosis for focal hepatic lesions.

Terminology

SPIO agent is a liver-specific contrast agent that contains superparamagnetic iron oxide nanoparticles coated with a carboxydextran shell. Ferucarbotran is taken up by reticuloendothelial system (RES) cells in the liver, spleen, bone marrow, and lymph nodes.

Peer review

The effectiveness of ferucarbotran-enhanced MR imaging in the detection of hepatic lesions is known. The present study compares the diagnostic efficiency of three MR imaging sequences in patients with focal hepatic lesions. The scientific value of this manuscript is demonstrated.

REFERENCES

- 1 **Zavadsky KE**, Lee YT. Liver metastases from colorectal carcinoma: incidence, resectability, and survival results. *Am Surg* 1994; **60**: 929-933
- 2 **Poon RT**, Fan ST. Hepatectomy for hepatocellular carcinoma: patient selection and postoperative outcome. *Liver Transpl* 2004; **10**: S39-S45
- 3 **Schwartz M**. Liver transplantation in patients with hepatocellular carcinoma. *Liver Transpl* 2004; **10**: S81-S85
- 4 **Yamamoto J**, Okada S, Shimada K, Okusaka T, Yamasaki S, Ueno H, Kosuge T. Treatment strategy for small hepatocellular carcinoma: comparison of long-term results after percutaneous ethanol injection therapy and surgical resection. *Hepatology* 2001; **34**: 707-713
- 5 **Llovet JM**, Bruix J. Systematic review of randomized trials for unresectable hepatocellular carcinoma: Chemoembolization improves survival. *Hepatology* 2003; **37**: 429-442
- 6 **Larson RE**, Semelka RC. Magnetic resonance imaging of the liver. *Top Magn Reson Imaging* 1995; **7**: 71-81
- 7 **Ward J**, Baudouin CJ, Ridgway JP, Robinson PJ. Magnetic resonance imaging in the detection of focal liver lesions: comparison of dynamic contrast-enhanced TurboFLASH and T2 weighted spin echo images. *Br J Radiol* 1995; **68**: 463-470
- 8 **Baron RL**. Detection of liver neoplasms: techniques and outcomes. *Abdom Imaging* 1994; **19**: 320-324
- 9 **Pauleit D**, Textor J, Bachmann R, Conrad R, Flacke S, Layer G, Kreft B, Schild H. Hepatocellular carcinoma: detection with gadolinium- and ferumoxides-enhanced MR imaging of the liver. *Radiology* 2002; **222**: 73-80
- 10 **Reimer P**, Rummeny EJ, Daldrup HE, Balzer T, Tombach B, Berns T, Peters PE. Clinical results with Resovist: a phase 2 clinical trial. *Radiology* 1995; **195**: 489-496
- 11 **Kopp AE**, Laniado M, Dammann F, Stern W, Grönewäller E, Balzer T, Schimpfky C, Claussen CD. MR imaging of the liver with Resovist: safety, efficacy, and pharmacodynamic properties. *Radiology* 1997; **204**: 749-756
- 12 **Josephson L**, Lewis J, Jacobs P, Hahn PF, Stark DD. The effects of iron oxides on proton relaxivity. *Magn Reson Imaging* 1988; **6**: 647-653
- 13 **Hamm B**, Staks T, Taupitz M, Maibauer R, Speidel A, Huppertz A, Frenzel T, Lawaczek R, Wolf KJ, Lange L. Contrast-enhanced MR imaging of liver and spleen: first experience in humans with a new superparamagnetic iron oxide. *J Magn Reson Imaging* 1994; **4**: 659-668
- 14 **McLachlan SJ**, Morris MR, Lucas MA, Fisco RA, Eakins MN, Fowler DR, Scheetz RB, Olukotun AY. Phase I clinical evaluation of a new iron oxide MR contrast agent. *J Magn Reson Imaging* 1994; **4**: 301-307
- 15 **Weissleder R**, Stark DD, Engelstad BL, Bacon BR, Compton CC, White DL, Jacobs P, Lewis J. Superparamagnetic iron oxide: pharmacokinetics and toxicity. *AJR Am J Roentgenol* 1989; **152**: 167-173
- 16 **Matsuo M**, Kanematsu M, Itoh K, Ito K, Maetani Y, Kondo H, Kako N, Matsunaga N, Hoshi H, Shiraishi J. Detection of malignant hepatic tumors: comparison of gadolinium- and ferumoxide-enhanced MR imaging. *AJR Am J Roentgenol* 2001; **177**: 637-643
- 17 **Denys A**, Arrive L, Servois V, Dubray B, Najmark D, Sibert A, Menu Y. Hepatic tumors: detection and characterization at 1-T MR imaging enhanced with AMI-25. *Radiology* 1994; **193**: 665-669
- 18 **Blakeborough A**, Ward J, Wilson D, Griffiths M, Kajiya Y, Guthrie JA, Robinson PJ. Hepatic lesion detection at MR imaging: a comparative study with four sequences. *Radiology* 1997; **203**: 759-765
- 19 **Shamsi K**, Balzer T, Saini S, Ros PR, Nelson RC, Carter EC, Tollerfield S, Niendorf HP. Superparamagnetic iron oxide particles (SH U 555 A): evaluation of efficacy in three doses for hepatic MR imaging. *Radiology* 1998; **206**: 365-371
- 20 **Thornbury JR**. Eugene W. Caldwell Lecture. Clinical efficacy of diagnostic imaging: love it or leave it. *AJR Am J Roentgenol* 1994; **162**: 1-8
- 21 **Ros PR**, Freeny PC, Harms SE, Seltzer SE, Davis PL, Chan TW, Stillman AE, Muroff LR, Runge VM, Nissenbaum MA. Hepatic MR imaging with ferumoxides: a multicenter clinical trial of the safety and efficacy in the detection of focal hepatic lesions. *Radiology* 1995; **196**: 481-488
- 22 **Okon E**, Pouliquen D, Okon P, Kovaleva ZV, Stepanova

- TP, Lavit SG, Kudryavtsev BN, Jallet P. Biodegradation of magnetite dextran nanoparticles in the rat. A histologic and biophysical study. *Lab Invest* 1994; **71**: 895-903
- 23 **Reimer P**, Allkemper T, Matuszewski L, Balzer T. Contrast-enhanced 3D-MRA of the upper abdomen with a bolus-injectable SPIO (SH U 555 A). *J Magn Reson Imaging* 1999; **10**: 65-71
- 24 **Lutz AM**, Willmann JK, Goepfert K, Marincek B, Weishaupt D. Hepatocellular carcinoma in cirrhosis: enhancement patterns at dynamic gadolinium- and superparamagnetic iron oxide-enhanced T1-weighted MR imaging. *Radiology* 2005; **237**: 520-528
- 25 **Jung CW**, Jacobs P. Physical and chemical properties of superparamagnetic iron oxide MR contrast agents: ferumoxides, ferumoxtran, ferumoxsil. *Magn Reson Imaging* 1995; **13**: 661-674
- 26 **Paley MR**, Mergo PJ, Torres GM, Ros PR. Characterization of focal hepatic lesions with ferumoxides-enhanced T2-weighted MR imaging. *AJR Am J Roentgenol* 2000; **175**: 159-163
- 27 **Imai Y**, Murakami T, Yoshida S, Nishikawa M, Ohsawa M, Tokunaga K, Murata M, Shibata K, Zushi S, Kurokawa M, Yonezawa T, Kawata S, Takamura M, Nagano H, Sakon M, Monden M, Wakasa K, Nakamura H. Superparamagnetic iron oxide-enhanced magnetic resonance images of hepatocellular carcinoma: correlation with histological grading. *Hepatology* 2000; **32**: 205-212
- 28 **Kim SH**, Choi D, Kim SH, Lim JH, Lee WJ, Kim MJ, Lim HK, Lee SJ. Ferucarbotran-enhanced MRI versus triple-phase MDCT for the preoperative detection of hepatocellular carcinoma. *AJR Am J Roentgenol* 2005; **184**: 1069-1076

S- Editor Ma N L- Editor Boyes A E- Editor Ma WH

Endoscopic management of gastrointestinal smooth muscle tumor

Xiao-Dong Zhou, Nong-Hua Lv, Hong-Xia Chen, Chong-Wen Wang, Xuan Zhu, Ping Xu, You-Xiang Chen

Xiao-Dong Zhou, Nong-Hua Lv, Chong-Wen Wang, Xuan Zhu, Ping Xu, You-Xiang Chen, Department of Gastroenterology, First Affiliated Hospital of Nanchang University, Nanchang 330006, Jiangxi Province, China

Hong-Xia Chen, Department of Gynecology and Obstetrics, First Affiliated Hospital of Nanchang University, Nanchang 330006, Jiangxi Province, China

Correspondence to: Dr. Xiao-Dong Zhou, Department of Gastroenterology, First Affiliated Hospital of Nanchang University, Nanchang 330006, Jiangxi Province, China. zhouxd7612@hotmail.com

Telephone: +86-791-8692540 Fax: +86-791-8623153

Received: May 25, 2007 Revised: June 18, 2007

2 cm. The "digging" biopsy technique would be a good option for histologic diagnosis of SMTs.

© 2007 WJG. All rights reserved.

Key words: Gastrointestinal; Smooth muscle tumor; Endoscopy; Endoscopic ultrasonography; Management

Zhou XD, Lv NH, Chen HX, Wang CW, Zhu X, Xu P, Chen YX. Endoscopic management of gastrointestinal smooth muscle tumor. *World J Gastroenterol* 2007; 13(36): 4897-4902

<http://www.wjgnet.com/1007-9327/13/4897.asp>

Abstract

AIM: To systematically evaluate the efficacy and safety of endoscopic resection of gastrointestinal smooth muscle tumors (SMTs, including leiomyoma and leiomyosarcoma) and to review our preliminary experiences on endoscopic diagnosis of gastrointestinal SMTs.

METHODS: A total of 69 patients with gastrointestinal SMT underwent routine endoscopy in our department. Endoscopic ultrasonography (EUS) was also performed in 9 cases of gastrointestinal SMT. The sessile submucosal gastrointestinal SMTs with the base smaller than 2 cm in diameter were resected by "pushing" technique or "grasping and pushing" technique while the pedunculated SMTs were resected by polypectomy. For those SMTs originating from muscularis propria or with the base size ≥ 2 cm, ordinary biopsy technique was performed in tumors with ulcers while the "Digging" technique was performed in those without ulcers.

RESULTS: 54 cases of leiomyoma and 15 cases of leiomyosarcoma were identified. In them, 19 cases of submucosal leiomyoma were resected by "pushing" technique and 10 cases were removed by "grasping and pushing" technique. Three cases pedunculated submucosal leiomyoma were resected by polypectomy. No severe complications developed during or after the procedure. No recurrence was observed. The diagnostic accuracy of ordinary and the "Digging" biopsy technique was 90.0% and 94.1%, respectively.

CONCLUSION: Endoscopic resection is a safe and effective treatment for leiomyomas with the base size \leq

INTRODUCTION

Gastrointestinal smooth muscle tumors (SMTs, including leiomyoma and leiomyosarcoma) represent relatively common lesions that are thought to originate from a muscular layer of gastrointestinal tract. They can be found in the esophagus, stomach, small intestine, and colon^[1-3]. The most common symptoms of gastrointestinal SMTs are gastrointestinal bleeding, as a result of overlaying mucosa ulceration, and pain. Other symptoms may include anorexia, dysphagia, obstruction, perforation, or fever^[4-6].

Gastrointestinal SMTs are difficult-to-cure gastrointestinal tumors when compared with polyps and the complete surgical resection is still considered to be the most definitive therapy for gastrointestinal SMTs. In recent years, several reports^[7-10] suggest that endoscopic treatment of GI submucosal leiomyoma is a valid alternative to invasion surgery. However, these reports cannot provide enough convincing evidence for the efficiency and safety of the treatment they used because lack of enough cases (majority of these reports include only one single case). Meanwhile, the endoscopic resection is inappropriate for leiomyosarcoma and those leiomyomas with either the base ≥ 2 cm in diameter or originating from muscularis propria because of the risk of hemorrhage and perforation^[7-10]. Therefore, a safe and efficient therapeutic strategy for endoscopic resection of leiomyoma is worth being explored.

From 1986-2006, more than 100 cases of gastrointestinal submucosal tumors (including leiomyoma) were found and successfully resected under endoscopy in our unit. Enlightened by these cases, we prospectively explored the feasibility, efficacy, and safety for endoscopic removal of

leiomyoma. During the last fifteen years, 69 cases of SMTs have undergone the endoscopic examinations and finally proven pathologically at our hospital. Within these, 32 cases of submucosal leiomyoma were successfully removed under endoscopy. The present study evaluated the efficacy and safety of our technique for endoscopic resection of submucosal leiomyoma. Meanwhile, our preliminary experience on diagnosis of SMT based on endoscopy and endoscopic ultrasonography (EUS) was also reviewed in this study.

MATERIALS AND METHODS

Patients

From January 1992 to January 2006, 69 cases of SMT were found under endoscopy and identified by further pathological examination at First Affiliated Hospital of Nanchang University (Nanchang, China). Of these, 39 were male and 30 were female. The age range was 15-74 (average 45.6) years. All the patients complained of at least one of the GI symptoms such as gastrointestinal bleeding, abdominal pain, anorexia, and dysphagia, which could be attributed to the SMT. Written informed consent was obtained from every patient. The locations and types of these SMTs are presented in Table 1. Under immunohistochemical staining, all these SMTs were positive for smooth muscle actin (SMA) but negative for CD117 (C-kit).

All the patients underwent routine gastrointestinal endoscopy (Olympus GF/CF 230 or 240I; PENTAX-2901) to assess the location, appearance, extent, and overlaying mucosa integrity of the SMTs. After February 2005, endoscopic ultrasonography (EUS, Olympus GF-UM20) was utilized to detect the size and shape, echodensity, and the originating layer of tumor in the wall structure. Interpretation was based on the five-layer structure of the wall. For those SMTs with overlaying mucosa ulcerations, the biopsy specimens were obtained from the bottom of the ulcer. The "digging" biopsy technique was employed for those protrusive lesions without overlaying mucosa ulceration but with the base ≥ 2 cm in diameter or originating from muscularis propria.

The criterion for choice of therapy was: (1) the pedunculated submucosal SMTs with the base smaller than 2 cm in diameter were resected by polypectomy; (2) the sessile submucosal SMTs with the base smaller than 2 cm in diameter were removed using a "pushing" technique or "grasping and pushing" technique; (3) those SMTs pathologically identified malignant, originated from muscularis propria, or with the base size ≥ 2 cm were surgically resected. Histopathologic features of both endoscopically and surgically removed SMTs were reviewed by two experienced histopathologists. In addition, all specimens underwent immunostaining of SMA and CD117 (C-kit). Histological examination was also used to determine whether the tumor was removed completely.

After endoscopic removal of SMT, patients were required to remain in the hospital for at least 2 d. Bed rest was necessary for the patients with colonic SMT. Patients with upper gastrointestinal SMTs fasted for 2 d. Endoscopy was performed one week after resection to

Table 1 The sites of the GI SMT identified by endoscopy and histological examination

	Location	Leiomyoma	Leiomyosarcoma
Esophageal	Upper	4	0
	Middle	11	0
	Lower	13	0
Stomach	Cadiac	4	0
	Fornix	4	6
	Corpus	5	4
	Antrum	6	2
Duodenum	Bulb	2	1
	Descending part	1	2
Colon	Ascending colon	2	0
	Transverse colon	2	0
Total		54	15

assess healing and examine hemorrhagic signs such as exposed vessels. Follow-up endoscopic examination was performed every six months for the first year and annually thereafter. Each case was followed up by endoscopic examination for 1-2 years.

Polypectomy

The technique for the resection of pedunculated SMTs was the same as initiated for epithelial polyps. In brief, the snare was placed around the stalk of the SMT, tightened and lifted toward the cavity of the GI tract. The snare was tightened gradually and the SMT was resected by coagulation current.

"Pushing" technique

For a sessile SMT, the snare was placed around the lesion (Figure 1). The head (gastroscopy) or anal (colonoscopy) side of the lesion was pushed by the insulated cannula of snare to form a semipedunculation. The snare was tightened gradually at the top of the semipedunculation and total SMT was captured and then resected completely by a high-frequency electrosurgical current.

"Grasping and pushing" technique

This technique was performed with a double channel endoscopy. In brief, a polypectomy snare inserted through the accessory channel was first placed around the submucosal tumor. The body of the tumor was lifted by a grasping forceps inserted from the other channel to form a semipedunculation. The submucosal tumor was then captured by tightening the snare gradually at the top of the semipedunculation. Finally, the tumor was resected by a high-frequency electrosurgical current.

"Digging" biopsy technique

Initially, a biopsy forceps was used to open a hole in the overlaying mucosa leaving the SMT exposed. At least 4 biopsy specimens were then obtained from the exposed SMT.

RESULTS

During the last 15 years, 54 cases of leiomyoma were

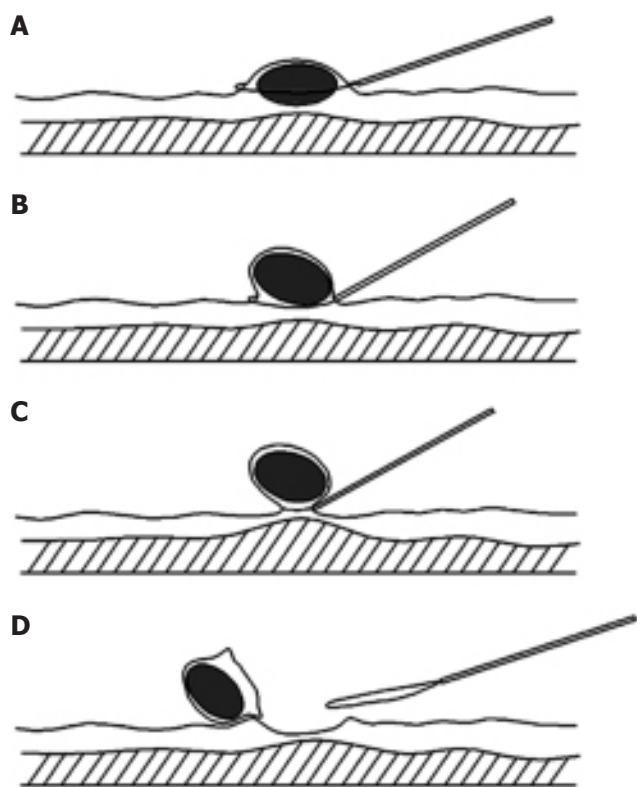


Figure 1 Schematic diagram for "pushing" technique. **A:** The snare was placed around the leiomyoma; **B:** The head (gastroscopy) or anal (colonoscopy) side of leiomyoma was pushed by the insulated cannula of snare to form a semipedunculation; **C:** The snare was tightened gradually and total leiomyoma was captured; **D:** The leiomyoma was resected completely.

identified at our hospital. Under endoscopy, most leiomyomas appeared as a red and smooth sessile protrusions with normal overlying mucosa. Only 3 cases of leiomyoma were shown as pedunculated lesions. The esophagus was the most frequent site (51.8%) for leiomyoma (Table 1), followed by stomach (35.2%), colon (7.4%), and duodena (5.6%). No correlation was found between the occurrence and either age or gender in this study. The average base size of leiomyomas measured under endoscopy was 1.2 ± 0.2 cm (standard deviation, SD). The smallest one was 0.8 cm in diameter while the largest was 3.4 cm in diameter. From February 2005 to January 2006, EUS was performed in 6 cases of leiomyoma. All these leiomyoma were shown as an echolucent mass with sharp margin and originating from muscularis mucosa (3 cases) or muscularis propria (3 cases) of gastrointestinal tract. Average size of these 6 cases of leiomyomas measured under EUS was 1.1 ± 0.3 cm (SD).

Among the above-mentioned 54 cases of leiomyoma, 19 cases of submucosal leiomyoma were resected by "pushing" technique (Figure 2) while 10 cases were removed by "grasping and pushing" technique (Table 2). Only 3 cases pedunculated submucosal leiomyoma were resected by polypectomy (Table 2). All these resected leiomyomas were confirmed by the following histopathologic examination. Immediate endoscopic observation after all these resections showed a 1.2-1.5 cm cauterization burn without other abnormalities. No complications such as perforation and hemorrhage

Table 2 The amount and location of the GI leiomyomas resected by "pushing" technique, "grasping and pushing" technique, and polypectomy, respectively

	Location	Pushing	Pushing and grasping	Polypectomy
Esophageal	Upper	2	0	0
	Middle	5	2	0
	Lower	3	3	1
Stomach	Cadiac	1	1	0
	Fornix	1	1	0
	Corpus	1	0	1
	Antrum	3	0	1
Duodenum	Bulb	0	2	0
	Descending part	0	1	0
Colon	Ascending colon	1	0	0
	Transverse colon	2	0	0
Total		19	10	3

developed during or after the procedure in most of these patients. Oozy bleeding occurred in 4 patients and easily controlled after epinephrine or thrombin spraying. After a follow-up period of one to two years with repeated endoscopy, no recurrence was found.

Among the remaining 22 cases of leiomyoma with the base size ≥ 2 cm or originating in muscularis propria, 9 cases were observed with occurrence of overlying mucosa ulceration. Of these, 8 cases (88.9%) were confirmed pathologically by obtaining biopsy specimens from the bottom of the ulcer while one case failed to report by this method. For those leiomyomas without ulcer, 12 cases (92.3%) were confirmed by "digging" biopsy while only one case (7.7%) failed to report by this method. All 22 cases of leiomyoma were successfully removed by surgery.

In this study, 15 cases of leiomyosarcoma were surgically resected and confirmed by following histopathologic examination. No correlation was found between the occurrence and either age or gender in this study. Of these, endoscopy revealed the lesion as an intraluminal protuberant tumour with ulcer (Figure 3A) in 7 cases and without ulcer in 4 cases. Another 4 cases appeared as an ulcer alone. The occurrence frequency of ulcer in leiomyosarcoma is 73.3% (11/15), which is obviously higher than that in leiomyoma (16.7%, 9/54). Leiomyosarcoma were observed in stomach (80.0%) and duodena (20.0%). The average base size of leiomyosarcoma measured under endoscopy was 6.8 ± 2.3 cm (SD). A significant difference was found between the base size of leiomyoma and leiomyosarcoma ($P < 0.001$, non-paired *t* test). From February 2005 to January 2006, three cases of leiomyosarcoma underwent EUS examination. All three tumours were found to arise from the fourth echo poor layer (muscularis propria); EUS showed that one gastric tumour disrupted all the wall layers. The tumour echostructure and margins were inhomogeneous and irregular in all three cases (Figure 3B).

In those 11 cases of leiomyosarcoma with occurrence of ulcer, 10 cases (90.9%) were confirmed pathologically by obtained biopsy at the bottom of the ulcer. One case failed to report by this technique and finally confirmed

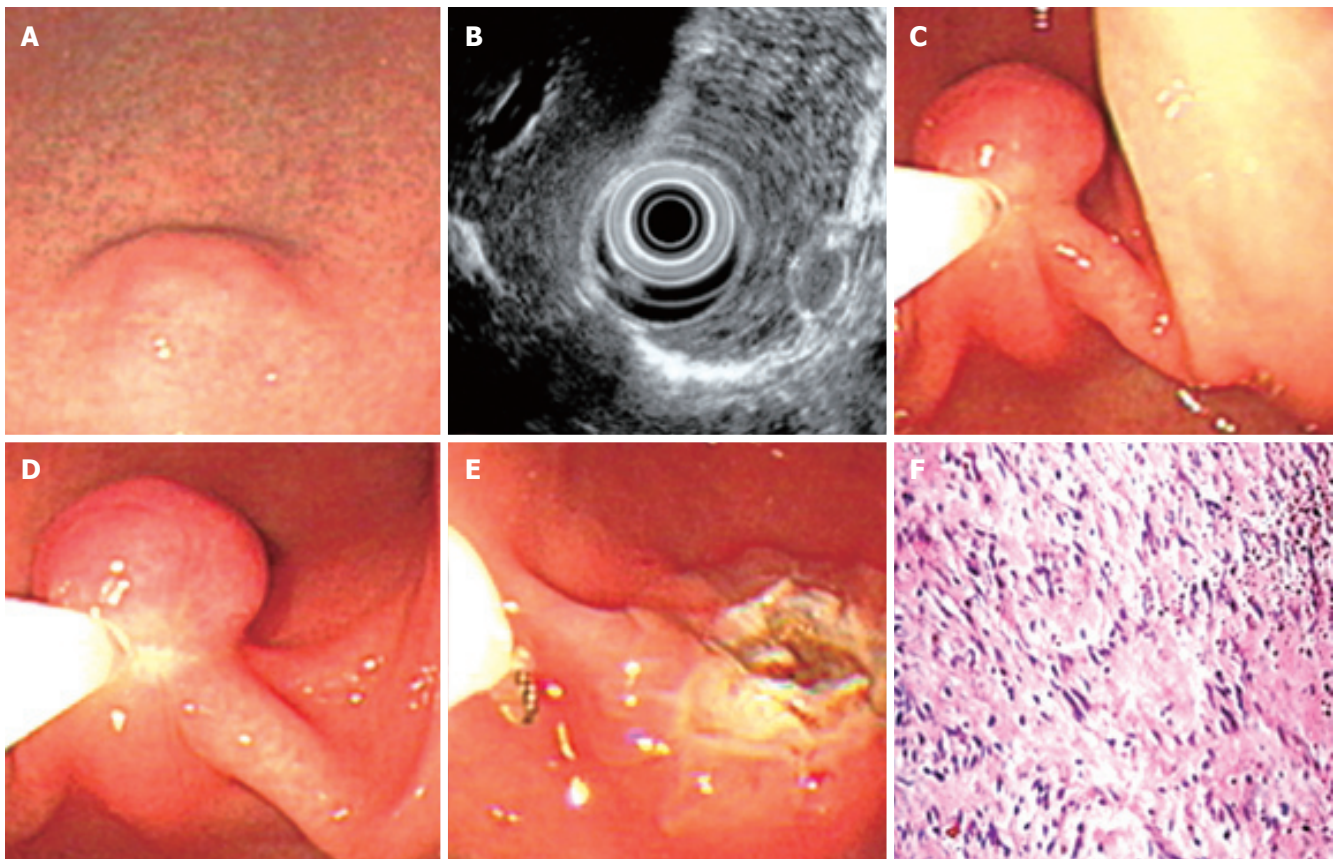


Figure 2 Endoscopic views for "pushing" resection of a leiomyoma. **A:** A sessile leiomyoma at antrum of stomach; **B:** EUS revealed that the mass originated from muscularis mucosa; **C:** The leiomyoma was pushed by cannula to form a semipedunculation and then captured by snare; **D:** The captured leiomyoma was resected by high-frequency electrosurgical current; **E:** The endoscopic view for the cauterization burn of leiomyoma after resection; **F:** The histologic view of leiomyoma after resection (HE, x 200).

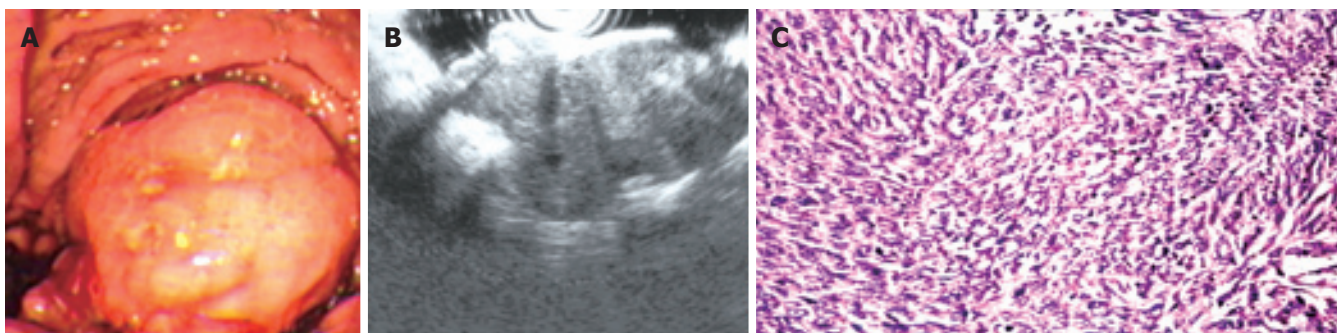


Figure 3 **A:** Endoscopic view of a leiomyosarcoma at corpus of stomach; **B:** The view of EUS; **C:** The histologic examination after "digging" technique (HE, x 200).

after surgical resection. The remaining 4 cases of leiomyosarcoma without ulcer were confirmed by "digging" biopsy.

DISCUSSION

To resect submucosal SMT by endoscopy, it is crucial to determine the originating layer of the lesion. If the tumor arises from muscularis propria, complete resection should be avoided because of the risk of perforation^[7-12]. Recently, EUS was considered to be very helpful in determining the size, consistency, extension of submucosal tumors, and the layer from which tumors originate^[12-15].

Therefore, the assistance of EUS greatly increases the safety of endoscopic resection of submucosal SMTs^[9,12,13]. In our unit, EUS were employed in evaluating 6 cases of leiomyoma. Of these, three cases were found originating from muscularis mucosa, and then were resected completely under endoscopy. Another 3 cases, originating from muscularis propria, were successfully performed via surgical resection. However, the EUS were equipped in our department only after January 2005. Before 2005, we determined the location of submucosal tumor through detecting the mobility of tumor by closed biopsy forceps. In brief, if the forceps pushed the tumor to slide under mucosa, it suggested that the tumor originated superficially

in muscularis mucosa and was resectable by means of endoscopy. Whereas, an immobile tumor revealed that the tumor had its roots in muscularis propria and could not be removed by endoscopic resection. Although we admit that this criterion is somewhat imprecise, all submucosal leiomyomas (26 cases) determined by this method were successfully performed endoscopic resections, no perforations or severe bleeding occurred after resection. In addition, in those 6 cases examined by EUS, the conclusions drawn by this method fit well with those by EUS. All these were enough to prove the reliability of our method.

The “Pushing” technique has been employed by our group for resection for various gastrointestinal submucosal tumors including leiomyoma, fibroma, lipoma, carcinoid. All these submucosal tumors were successfully resected, with the exception of one case of fibroma, which underwent severe bleeding after resection. In the present study, 19 cases of leiomyoma were successfully and safely removed by the “pushing” technique. No recurrence was observed after 1-2 years follow-up. The crucial step for this technique is the movement of pushing. Because of the pushing by insulated cannula of snare, a semipedunculation forms and then the whole body of the tumor is easily captured by pre-placed snare. This ensures that the tumor can be resected completely. Meanwhile, because the tumor body is lifted toward cavity before cauterization and the line of resection is at the bottom of the tumor body and the top of the semipedunculation, the muscularis propria has already separated from the place of cauterization and then will not be injured by the high-frequency electrosurgical current. Additionally, to minimize potential severe hemorrhage and perforation, we avoid undergoing endoscopic resection of those lesions with the base ≥ 2 cm. All these fully demonstrate the efficiency and safety of the “pushing” technique utilized. When compared with other techniques such as En Bloc^[9,15-17], the pushing technique is much easier to be operated and takes less time.

The En bloc technique has been used by several groups for endoscopic resection of submucosal SMTs^[9,15-17]. By our understanding, there are at least two advantages for this technique. First, this technique contains an important step-injection of the saline solution into the submucosa, which is to separate the line of resection from muscularis propria and then prevent the injury of muscle layer. This step greatly increases the safety of the operation. Second, the removed tumors can be easily captured for further histological examination. However, we consider that this technique also has the shortcomings of complicated operation and time-consuming. In fact, it is a tough job for an endoscopist to inject the solution exactly into the base of the leiomyoma without injuring the wall of the gastrointestinal tract. Furthermore, injection of saline may make the margin of the lesion unclear. To overcome these problems, we tried to explore the possibility of deleting the procedure of saline injection. Normally, 19.4 mm grasping forceps can easily grasp the body of tumor and lift it up towards the cavity of the gastrointestinal tract to form a pseudo-pedunculation. In fact, this step has already separated the tumor from muscularis propria and then is

enough to prevent the injury of muscle layer when the captured tumor is removed by pre-placed snare. In some cases, the surface of the tumor is too slippery and difficult to grasp. To solve this problem, we first grasped the overlaying mucosa, and then pushed the tumor by insulated cannula of snare to form a semipedunculation, which also prevented the injury of muscularis propria when captured tumors were removed by high-frequency electrosurgical current. In the last 15 years, 10 cases of leiomyoma have been safely removed in our department by using this “grasping and pushing” technique. No recurrence was observed after 1-2 years follow-up. All these fully support the efficiency and safety of the grasping and pushing technique we utilized.

For those SMTs with the base size ≥ 2 cm and/or originating in muscularis propria, to differentiate malignant from benign is crucial for further treatment. Histological diagnosis is necessary not only to ascertain whether a lesion is benign or malignant (usually larger lesions with irregular borders, inhomogeneous areas, or eroded surfaces), but also to detect smaller lesions without malignant morphologic features. In recent years, several methods have been developed for this histological diagnosis. Matsui *et al.*^[18] have described a biopsy technique-endoscopic ultrasonography-guided fine needle aspiration biopsy (EUS-FNAB). In this technique, the biopsy materials are obtained from a needle, which is inserted into the lesions guided by EUS. Open biopsy, developed by Kojima *et al.*^[9], is another effective biopsy technique for gastrointestinal submucosal lesions. In this technique, the covering mucosa is resected to expose the tumor and then several tissues are obtained by ordinary forceps at the bottom of the artificial ulcer. The techniques we used in this study were the “digging” and ordinary biopsy techniques. In order to evaluate the diagnostic accuracy of these two techniques, we selected those cases which were finally removed by surgery. In our series, the diagnostic accuracy of “digging” biopsy is 94.1% for those SMTs without ulcer (leiomyoma: 92.3%; leiomyosarcoma: 100%). This result is very close to the above-mentioned two techniques, but the “digging” biopsy is much easier and cheaper than EUS-FNAB and open biopsy. In this series, no severe hemorrhage developed after the “digging” biopsy. In addition, the diagnostic accuracy of ordinary biopsy in the SMTs with ulcer is 90.0%, similar to that of “digging” biopsy.

Although EUS is very helpful in deciding the technique for endoscopic resection of submucosal SMTs, it is difficult to differentiate the malignant from benign SMTs by means of EUS unless there is local extension or metastasis, because no significant difference has been found between malignant and benign lesions with regard to homogeneity of internal echo pattern or marginal echo pattern^[3,19,20]. However, EUS is considered to be reliable in predicting the potential malignancy of SCTs^[3]. The three most predictive EUS features described by Palazzo *et al.*^[3] are irregular margins, cystic spaces, and lymph nodes with a malignant patterns. Palazzo *et al.*^[3] concluded that (1) the presence of at least one of these criteria had a sensitivity of 91%, a specificity of 88%, a positive predictive value of 83%, and a negative predictive value of 94% for

potential malignancy; (2) a combination of two of these three criteria had a positive predictive value and specificity of 100%; (3) tumors of 30 mm or less, with regular extraluminal margins and a homogeneous pattern, are likely to be benign. Our series also support predictive EUS features although only 6 cases of leiomyoma and 3 cases of leiomyosarcoma were investigated.

In conclusion, endoscopic resection is a safe and effective therapy for submucosal leiomyoma with the base size ≤ 2 cm. The guidance of EUS greatly increases the safety of endoscopic resection of submucosal leiomyoma. The “digging” biopsy technique would be a good option for the histologic diagnosis of SMT.

COMMENTS

Background

Gastrointestinal smooth muscle tumors (SMTs, including leiomyoma and leiomyosarcoma) represent relatively common lesions. The complete surgical resection was considered to be the most definitive therapy for SMTs in the past. In recent years, some researchers reported that endoscopic treatment of GI submucosal leiomyoma is a valid alternative to invasion surgery. However, they failed to provide enough convincing evidences for the efficiency and safety of the treatment they used because lack of enough cases.

Research frontiers

In the last few decades, gastrointestinal endoscopy has been widely used in the treatment of gastrointestinal diseases, it is very important to elucidate the efficiency and safety of endoscopic treatment of SMTs.

Innovations and breakthroughs

The authors revealed that endoscopic treatment of SMTs is efficient and safe through a prospective research with many cases. Meanwhile, the “Pushing” technique and “Grasping and pushing” technique were put forward and analyzed.

Applications

The current study will guide the clinical application of endoscopic treatment of SMTs.

Peer review

This paper may show us endoscopic management of smooth muscle tumor in a single hospital for more than 10 years. However, this study is only a descriptive study of the experienced cases. The authors should consider again the novel findings obtained from the experienced cases.

REFERENCES

- Miettinen M, Sarlomo-Rikala M, Sobin LH, Lasota J. Esophageal stromal tumors: a clinicopathologic, immunohistochemical, and molecular genetic study of 17 cases and comparison with esophageal leiomyomas and leiomyosarcomas. *Am J Surg Pathol* 2000; **24**: 211-222
- Chak A, Canto MI, Rösch T, Dittler HJ, Hawes RH, Tio TL, Lightdale CJ, Boyce HW, Scheiman J, Carpenter SL, Van Dam J, Kochman ML, Sivak MV. Endosonographic differentiation of benign and malignant stromal cell tumors. *Gastrointest Endosc* 1997; **45**: 468-473
- Palazzo L, Landi B, Cellier C, Cuillerier E, Roseau G, Barbier JP. Endosonographic features predictive of benign and malignant gastrointestinal stromal cell tumours. *Gut* 2000; **46**: 88-92
- Tricarico A, Cione G, Sozio M, Di Palo P, Bottino V, Martino A, Tricarico T, Falco P. Digestive hemorrhages of obscure origin. *Surg Endosc* 2002; **16**: 711-713
- Campbell F, Bogomoletz WV, Williams GT. Tumours of the oesophagus and stomach. In: Fletcher CD. Diagnostic histopathology of tumours. London: Churchill Livingstone, 1995: 193-242
- Kempson RL, Hendrickson MR. Gastrointestinal stromal (smooth muscle) tumours. In: Whitehead R, editor. Gastrointestinal and oesophageal pathology. 2nd ed. Edinburgh: Churchill Livingstone, 1995: 727-739
- Inoue H, Kawano T, Tani M, Takeshita K, Iwai T. Endoscopic mucosal resection using a cap: techniques for use and preventing perforation. *Can J Gastroenterol* 1999; **13**: 477-480
- Yu JP, Luo HS, Wang XZ. Endoscopic treatment of submucosal lesions of the gastrointestinal tract. *Endoscopy* 1992; **24**: 190-193
- Kojima T, Takahashi H, Parra-Blanco A, Kohsen K, Fujita R. Diagnosis of submucosal tumor of the upper GI tract by endoscopic resection. *Gastrointest Endosc* 1999; **50**: 516-522
- Lee IL, Lin PY, Tung SY, Shen CH, Wei KL, Wu CS. Endoscopic submucosal dissection for the treatment of intraluminal gastric subepithelial tumors originating from the muscularis propria layer. *Endoscopy* 2006; **38**: 1024-1028
- Chow WH, Kwan WK, Ng WF. Endoscopic removal of leiomyoma of the colon. *Hong Kong Med J* 1997; **3**: 325-327
- Sun S, Wang M, Sun S. Use of endoscopic ultrasound-guided injection in endoscopic resection of solid submucosal tumors. *Endoscopy* 2002; **34**: 82-85
- Waxman I, Saitoh Y, Raju GS, Watari J, Yokota K, Reeves AL, Kohgo Y. High-frequency probe EUS-assisted endoscopic mucosal resection: a therapeutic strategy for submucosal tumors of the GI tract. *Gastrointest Endosc* 2002; **55**: 44-49
- Oğuz D, Filik L, Parlak E, Dişibeyaz S, Çiçek B, Kaçar S, Aydoğ G, Sahin B. Accuracy of endoscopic ultrasonography in upper gastrointestinal submucosal lesions. *Turk J Gastroenterol* 2004; **15**: 82-85
- Hunt GC, Rader AE, Faigel DO. A comparison of EUS features between CD-117 positive GI stromal tumors and CD-117 negative GI spindle cell tumors. *Gastrointest Endosc* 2003; **57**: 469-474
- Higaki S, Hashimoto S, Harada K, Nohara H, Saito Y, Gondo T, Okita K. Long-term follow-up of large flat colorectal tumors resected endoscopically. *Endoscopy* 2003; **35**: 845-849
- Yamamoto H, Kawata H, Sunada K, Sasaki A, Nakazawa K, Miyata T, Sekine Y, Yano T, Satoh K, Ido K, Sugano K. Successful en-bloc resection of large superficial tumors in the stomach and colon using sodium hyaluronate and small-caliber-tip transparent hood. *Endoscopy* 2003; **35**: 690-694
- Matsui M, Goto H, Niwa Y, Arisawa T, Hirooka Y, Hayakawa T. Preliminary results of fine needle aspiration biopsy histology in upper gastrointestinal submucosal tumors. *Endoscopy* 1998; **30**: 750-755
- Rösch T, Lorenz R, Dancygier H, von Wickert A, Classen M. Endosonographic diagnosis of submucosal upper gastrointestinal tract tumors. *Scand J Gastroenterol* 1992; **27**: 1-8
- Sotoudehmanesh R, Ghafoori A, Mikaeli J, Tavangar SM, Moghaddam HM. Esophageal leiomyomatosis diagnosed by endoscopic ultrasound. *Endoscopy* 2005; **37**: 281

S- Editor Zhu LH L- Editor Li M E- Editor Ma WH

Does protracted antiviral therapy impact on HCV-related liver cirrhosis progression?

Giovanni Tarantino, Antonio Gentile, Domenico Capone, Vincenzo Basile, Marianna Tarantino, Matteo Nicola Dario Di Minno, Alberto Cuocolo, Paolo Conca

Giovanni Tarantino, Matteo Nicola Dario Di Minno, Paolo Conca, Department of Clinical and Experimental Medicine, Federico II University Medical School of Naples, Naples 80131, Italy

Antonio Gentile, Domenico Capone, Vincenzo Basile, Marianna Tarantino, Alberto Cuocolo, Department of Biomorphological and Functional Sciences, Pharmacology Section of Neuroscience Department, Federico II University Medical School of Naples, Naples 80131, Italy

Correspondence to: Giovanni Tarantino, MD, Department of Clinical and Experimental Medicine, Federico II University Medical School of Naples, Via S. Pansini, 5, Naples 80131, Italy. tarantin@unina.it

Telephone: +39-81-7462024 Fax: +39-81-5466152

Received: May 31, 2007 Revised: June 18, 2007

Abstract

AIM: To study the outcomes of patients with compensated hepatitis C virus-related cirrhosis.

METHODS: Twenty-four grade A5 and 11 grade A6 of Child-Pugh classification cirrhotic patients with active virus replication, treated for a mean period of 31.3 ± 5.1 mo with moderate doses of interferon-alpha and ribavirin, were compared to a cohort of 36 patients with similar characteristics, without antiviral treatment. Salivary caffeine concentration, a liver test of microsomal function, was determined at the starting and thrice in course of therapy after a mean period of 11 ± 1.6 mo, meanwhile the resistive index of splenic artery at ultra sound Doppler, an indirect index of portal hypertension, was only measured at the beginning and the end of study.

RESULTS: Eight out of the 24 A5- (33.3%) and 5 out of the 11 A6- (45.45%) treated-cirrhotic patients showed a significant improvement in the total overnight salivary caffeine assessment. A reduction up to 20% of the resistive index of splenic artery was obtained in 3 out of the 8 A5- (37.5%) and in 2 out of the 5 A6- (40%) cirrhotic patients with an improved liver function, which showed a clear tendency to decrease at the end of therapy. The hepatitis C virus clearance was achieved in 3 out of the 24 (12.5%) A5- and 1 out of the 11 (0.091%) A6-patients after a median period of 8.5 mo combined therapy. In the cohort of non-treated cirrhotic patients, not only the considered parameters remained unchanged, but 3 patients (8.3%) had a worsening of

the Child-Pugh score ($P = 0.001$).

CONCLUSION: A prolonged antiviral therapy with moderate dosages of interferon-alpha and ribavirin shows a trend to stable liver function or to ameliorate the residual liver function, the entity of portal hypertension and the compensation status at acceptable costs.

© 2007 WJG. All rights reserved.

Key words: Liver cirrhosis; Hepatitis C virus infection; Antiviral therapy

Tarantino G, Gentile A, Capone D, Basile V, Tarantino M, Di Minno MND, Cuocolo A, Conca P. Does protracted antiviral therapy impact on HCV-related liver cirrhosis progression? *World J Gastroenterol* 2007; 13(36): 4903-4908

<http://www.wjgnet.com/1007-9327/13/4903.asp>

INTRODUCTION

Hepatitis C virus (HCV) infection is a leading cause of liver disease worldwide. Infected patients usually develop chronic hepatitis, which may progress producing diffuse disorganization of normal hepatic structure, to liver cirrhosis over a long period. Approximately 10%-20% of patients with chronic HCV infection have cirrhosis at the first clinical presentation, and near 20%-30% of those who do not present liver cirrhosis will eventually develop this illness and its complications^[1-3].

To date, the most effective treatment to prevent disease progression and eventually cure chronic hepatitis C (CHC) virus infection is the combined therapy of pegylated (PEG) interferon (IFN) alpha and ribavirin (RBV). The rate of sustained viral response (SVR) in immunocompetent patients undergoing this antiviral regimen ranges from 42% to approximately 80%, depending on the HCV genotype, with an acceptable safety profile^[6,7]. Thus, PEG-IFN alpha has substituted standard IFN alpha in the regimens used to treat CHC. However, the advantage of such an approach, with the additional cost it entails, still remains to be demonstrated^[8]. In patients with compensated HCV-related cirrhosis, standard IFN alpha or combination therapy offers an interesting SVR^[9]. Neither substantial difference has been reported between PEG-IFN alpha and standard IFN alpha in combination with

RBV, nor PEG-IFN has a higher risk of complications^[10]. Indeed, also in the decompensated form of liver cirrhosis, favourable results have been achieved after a 24 wk-course of combined therapy^[11]. Since liver cirrhosis progression is related to other factors beyond the viral infection, the therapeutic efficacy should be evaluated under a wider perspective. Actually, data on the liver residual function and hepatic hemodynamics assessment, following the route of compensated liver cirrhosis after antiviral therapy, are limited, having prevalently studied the survival rate and hepato-carcinoma appearance.

Expecting a modification of the liver parenchymal structure and vascular bed (as partial gain following the reduced cellular damage, fibrosis and nodular regeneration) after a long-term antiviral treatment at a moderate-dosage, our principal aim was to investigate the impact of this schedule on eventually modifying the progression of liver cirrhosis. Then, we started weighing three characteristic prognostic aspects of the illness, i.e., the residual liver function (index of intact cellular mass) by a quantitative liver function test (QLFT), the presence of liver decompensation by regular assessment of Child-Pugh (C-P) severity class, and the altered hemodynamics (consequence of a cyto-architectural alteration) by a non-invasive parameter reflecting the portal hypertension entity.

MATERIALS AND METHODS

Population

Among the 121 patients (regularly followed up at our out-patient clinic) suffering from HCV-related liver cirrhosis, with HCV load > 800 000 IU/mL and genotype 1, 71 were enrolled in the study, well matched for sex, age and severity of the disease. These 71 patients were divided into treated group (35) and non-treated group (36) (Table 1). Of the patients (including 13 females) in the first group, 24 (mean age 58.9 ± 5.7 years) belonged to C-P grade A5 cirrhotics and 11 (mean age 61 ± 5.0 years, 7F) to C-P grade A6 cirrhotics. A further characteristic was that 18 underwent previous antiviral therapy during the past years (14 non-responders and 4 relapsers). These patients were treated for a mean period of 31.3 ± 5.1 mo with moderate doses of IFN alpha (8 with 1.5 MU daily, 13 with 3 MU on alternate days and 14 with 1 mcg/kg body weight of Peg-IFN alpha 2b weekly) in combination with oral RBV (400 or 600 mg per day for body weight < or > 75 kg). One was excluded from the study because he spontaneously ended the therapy and two had a suspension period of more than six weeks. Collaterally, a cohort (without antiviral treatment) of 36 initially compensated cirrhotics (25, A5 C-P patients including 12 females, and 11 including 6 females, A6 C-P patients) was studied for the same period (9 patients were suffering from various grades of mood disorders, 4 were affected by microcythemia, 6 presented with thyroid laboratory abnormalities, 17 refused the treatment by choice. Of these patients, 15 were non-responders and 2 relapsers to previous antiviral therapy).

No patient in the two groups received mono- or combined treatment in the last two years.

The diagnosis of liver cirrhosis was made by histology

(25 patients), laboratory data (34 patients), and combined clinical and laboratory parameters plus ultra sound (US) findings (12 patients).

Main outcome measures

As QLFT, we chose to probe the microsomal activity of enzymes, e.g., cytochrome P450 1A2, with the total overnight salivary caffeine assessment (TOSCA), comparable to the well-known caffeine clearance according to a recent study^[12] and with the normal values set at ≤ 1 mcg/mL. Briefly, patients were allowed to drink coffee in late afternoon and on the day after overnight intake-washout, a sample of saliva, was collected in the morning, roughly centrifuged, frozen and stored until analysis. TOSCA was performed at first and then thrice during the course of therapy, at a mean interval period of 11 ± 1.6 mo. Caffeine was determined by an enzyme multiplied immunoassay technique (Dade Behring Liederbach, Germany) at the end of the follow-up period. Clinical assessment combined with other laboratory parameters, i.e., serum albumin, prothrombin time and bilirubin levels, was performed between periods of TOSCA evaluation. Occult blood in stools was detected to exclude gastrointestinal haemorrhage due to portal hypertension, as a possible cause of decompensation.

The resistive index of the splenic artery (SARI) at US Doppler was used to indirectly ascertain the entity of portal hypertension as previously described^[13]. SARI was taken at the beginning and the end of the observation period using a 3.5 convex probe of an ESAOTE (Genoa, Italy), by two operators who were blind to each other and the laboratory results.

A slightly modified C-P classification was adopted to define the severity of liver cirrhosis (Table 2). A worsening total score was set at three points when patients belonged to the A5 class and two points in case of the A6 class.

Exclusion criteria

The exclusion criteria included: (a) alcohol abuse screening according to recent recommendation^[8], (b) metabolic syndrome following the adult treatment panel III classification^[9], (c) detection of iron overload, (d) serum positivity for HBsAg, (e) previous chronic use of potentially toxic drugs as amiodarone, methotrexate and high doses of vitamin A.

Statistical analysis

Chi square test was used to study the frequencies. Differences between means were studied by unpaired T test or Mann-Whitney test. Wilcoxon signed ranks test was carried out for paired observations. ANCOVA (repeated measure analysis of variance corrected for the basal values) was carried out to analyze the differences in TOSCA data at various time intervals. Association was evaluated by applying Pearson correlation. Logistic regression was used to verify if the IFN formulation and the schedule of antiviral therapy affected the severity of liver cirrhosis, based on C-P classification. The inter-observer agreement at US was appraised by the Cohen's Kappa. Kaplan-Meier (K-M) survival curves were used for comparing

Table 1 Laboratory and sonographic parameters of the population at entry

	Treated									
	Not (36 patients)					Yes (35 patients)				
	Mean	SD	Median	Minimum	Maximum	Mean	SD	Median	Minimum	Maximum
ALT ¹	61.25	9.7				61.3	13.6			
Chol ²			133.5	59	190			132	89	200
Plts ³			141	67	191			134	90	193
SLD at US ⁴			134	122	151			133	120	146

ALT: alanine aminotransferase; Chol: total cholesterol; Plts: platelets; SLD: splenic longitudinal diameter; US: ultra sound; SD: standard deviation. ¹*P* = 0.97 at unpaired T test; ²*P* = 0.72 at Mann-Whitney (M-W) test; ³*P* = 0.15, at M-W test; ⁴*P* = 0.65 at M-W test.

Table 2 Child-Pugh classification (slightly modified)

Clinical and laboratory data	Points for increasing abnormality ¹		
	1	2	3
HE (grade ²)	None	(SHE)0-1-2	3-4
Ascites	None at US	Mild or controlled by diuretics	Present despite diuretics
PT (% of activity) ³	> 70	70-40	< 40
Albumin (g/dL)	> 3.5	2.8-3.5	< 2.8
Bilirubin (mg/dL)	< 2	2-3	> 3

¹Scoring system: 5-6 points, grade A; 7-9 points, grade B; 10-15 points, grade C. ²HE: hepatic encephalopathy; Grade 0: sub-clinical hepatic encephalopathy, SHE, unravelled by retain tests A or B; Grade 1: anxiety, irritability, depression, impaired concentration, sleep disturbances; Grade 2: disorientation, poor short-term memory, disinhibited behaviour, drowsiness; Grade 3: somnolence, bizarre behaviour, confusion, amnesia, paranoia; Grade 4: Coma. ³PT, prothrombin time, also expressed in seconds prolonged < 4; 4-6; > 6, or as INR < 1.7; 1.7-2.3; > 2.3. US: ultra sound.

C-P classification worsening probability curves in the two groups of patients. Cox proportional-hazards regression was employed to analyze the effect of non treatment (risk factor) on the C-P score aggravation. MedCalc version 9.0.2.1. and SYSTAT version 11.00.01 were used.

The study was conducted following the International Conference on Harmonization for Good Clinical Practice, in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki).

RESULTS

Whenever neutrophil count was < 750 cells/ μ L or haemoglobin < 10 g/dL, granulocyte colony-stimulating factor (filgrastim, Neupogen, Amgen Inc., California, 300 μ g to eight patients, weekly) or erythropoietin analog (Eprex, Janssen-Cilag, 10 000 units to seven patients, twice/thrice weekly) was administered (after the blood count increased, the growth factor administration was prolonged for at least two weeks). In the treated group, six out of the 24 A5 C-P (25%) and four out of the 11 A6 C-P (36.4%) cirrhotic patients showed an improvement (> 20% in respect to the base-line determination) in TOSCA (Figure 1). A clear association was found between the post-treatment TOSCA values and the prothrombin time ($r = -0.76$, *P* = 0.0001). No patient showed a worsening liver function. In the comparative cohort of initially

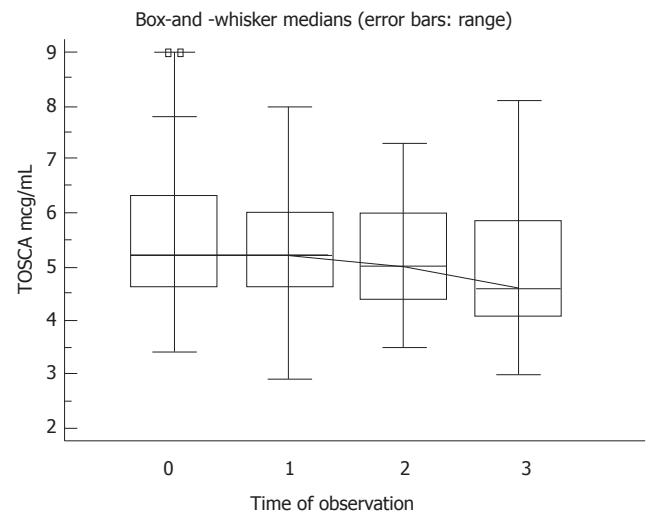


Figure 1 Behaviour of the applied quantitative liver function test in 35 patients treated with combined antiviral therapy. TOSCA: total overnight salivary caffeine assessment. The knurled line shows which groups are significantly different in the values of TOSCA, in respect to the basal ones; ANCOVA (repeated measure) test, *P* = 0.0001.

compensated cirrhotic patients without antiviral treatment, five out of the 36 patients (13.9%) had an exacerbation in respect to previous TOSCA values. A higher C-P score was found in non-treated than with treated patients (8/36 versus 1/35, *P* = 0.04).

Taking the US operator-dependent variability into consideration (although our inter-observer agreement was high, i.e., 89%), an appreciable reduction (> 20% compared with the pre-treatment measurements) in the SARI was obtained in 3 of 6 A5 C-P and 2 of 4 A6 C-P cirrhotic patients, showing an improvement in liver function. Furthermore, a significant decrease in the median SARI was present in the whole population compared to the pre- and post-treatment values.

HCV viral clearance was achieved in 3 out of the 24 (12.5%) A5 C-P and 1 out of the 11 (.91%) A6 C-P patients after a median (range) period of 8.5 (8-10) mo combined therapy.

No independent risk factor for C-P worsening was highlighted among the type of IFN, doses and period of treatment.

In our series during the entire period of study, no liver failure, hepato-cellular carcinoma and non-Hodgkin's lymphoma were discovered. There was a significant difference in the K-M curves of liver cirrhosis during

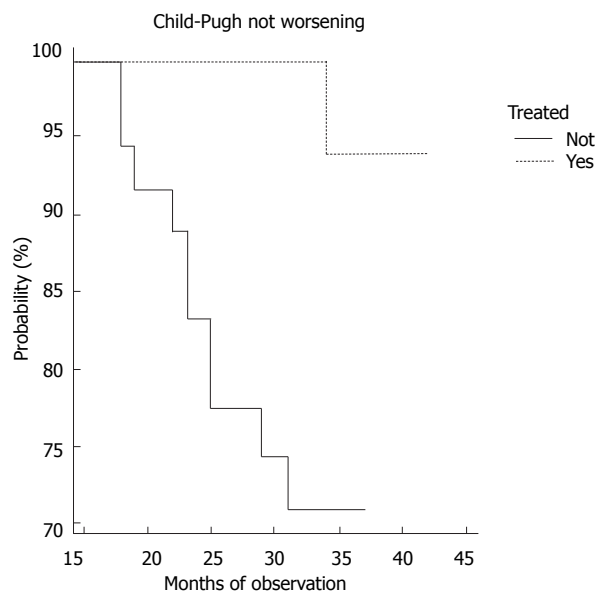


Figure 2 Behaviour of Child-Pugh classification in patients on (n 35) or not on (n 36) combined antiviral therapy. Comparison of the probability curves between treated and non-treated patients was made (Log-rank test). Chi-square test = 8.3, $P = 0.004$; Hazard ratio = 10.9; 95% CI = 1.75-18.9.

the four year-observation period between the two groups. About 30% of patients in the non-treated group experienced a worsening C-P score than the opposite cohort (Figure 2).

A small percentage of side-effects was registered in the treated group, e.g., two patients showed onset or enlargement of psoriatic plaques, three asthenia, one repeated low urinary tract infection, six anorexia, two low-grade fever, seven local erythema, one mild alopecia.

The cost of combined therapy plus growth factors per patient was estimated to be approximately 6000 US\$ for standard IFN and 15 000 US\$ for Peg-IFN, respectively.

The final values of TOSCA in samples were assessed for each group of patients ($\alpha = 0.05$, $\beta = 0.01$).

DISCUSSION

The main outcomes of antiviral therapy for chronic hepatitis C detected are the viral clearance and its lasting time. Actually, IFN therapy seems also be able to determine a decrease in the rate of functional disease progression in patients with the same disease, even in non-responders and relapsers^[14].

On the other hand, previous data have emphasised the scarce response to IFN therapy when dealing with advanced chronic liver diseases. This finding has not been further supported by a recent study^[11]. However, an uncertainty still exists, because it is not clear if a reduction in viral load or its clearance is of some benefit for any of the main processes, i.e., fibrosis and nodular regeneration, the extent of which determines the severity of liver cirrhosis. Variation exists among individuals regarding the rate and time of the progression of fibrosis to cirrhosis. There is evidence that the Ito cells are activated in response to HCV-induced hepatocellular

damage initiating fibrosis^[15]. These cells proliferate and become myofibroblasts, enhancing degradation of the normal matrix and producing excess abnormal matrix. Reactive oxygen species (ROS)^[16] and inflammatory cytokines (mainly transforming growth factor and platelet-derived growth factor) speed up fibrosis. These cells play a key role in the alteration of metalloproteinase enzymes that regulate matrix collagen metabolism^[17]. Fibrous tracts join branches of afferent portal veins and efferent hepatic veins, bypassing the hepatocytes and limiting their blood supply. Since the same myofibroblasts stimulated by endothelin-1, contribute to the increased portal vein resistance, fibrosis leads to "hepatocyte ischemia" with successive hepatocellular dysfunction and portal hypertension. Cirrhosis resulting from HCV infection has a slowly progressing course. Then, using the classic ones as therapeutic end-points^[18], could result in the short-medium ones. Furthermore, an eventual change in some prognostic aspects of liver cirrhosis has not been reported, especially if long-term, moderate-dosage schedules were used.

Having this in mind, we tracked the progression of liver cirrhosis, using three combined parameters, all indices of the severity of the illness.

We found an improvement in residual liver function as assessed by TOSCA, a lack of C-P classification variation and a concomitant constancy of the indirect assessment of portal hypertension grade. All of them showed a stable severity of the illness in treated patients. Antiviral therapy could affect the ongoing liver cirrhosis by influencing the inflammation- or apoptosis-mediated fibrosis process, working on HCV viral replication, lessening ROS formation. The last mechanism is of paramount importance because it alters reparative processes. Interestingly, a reduced cellular damage could also justify the improvement in QLFT following therapy. Obviously, SARI is a surrogate marker, reflecting the hyper-dynamic, high-flow circulatory state as a consequence of splanchnic vasodilatation caused by portal hypertension. Actually, the only supra-hepatic vein catheterization could ultimately give valid information on hypertensive status. In any case, this non-invasive tool offers its best resources for serial measurements of hypertension and it has not yet been criticized as other Doppler US parameters^[19]. In our study, a stable sonographic parameter or a slightly-reduced sonographic parameter which non invasively weighs the portal hypertension, was evidenced. Furthermore, the present study has other limitations in its methodology. The first is that it was not a randomized study, but a pilot study. In fact, only patients who accepted the treatment or had no contra-indications were enrolled. The second is that a four-year-interval was not representative of the natural history of this chronic disease, although it could foresee a certain trend. The third is that even if we tried to exclude the presence of co-factors, we were not sure that occult causes such as low intake of alcohol or episodic drug toxicity could have influenced the progression of liver cirrhosis in the non-treated patients. Moreover, we could have measured a serum fibrosis marker, a procollagen-III peptide, even if a consolidated study has failed to assess its utility^[20]. We stress that the follow-up and work-up

intervals were similar in the two groups. The fourth is that the quality of life was not opportunely evaluated, which is very important for the patient, although the non-standard dosage plays a key role in reducing side-effects. One positive aspect of this approach could be the health-care-cost in light of a likely procrastination of the need for hospitalization and liver transplantation.

In conclusion, our results favour the hypothesis that a stability (lack of fatal progression) or a trend to improve the severity of liver cirrhosis based on the clinical status and laboratory-instrumental profile is a reliable end-point after antiviral therapy. A prolonged combined antiviral course at reduced doses may slow down the progression of liver disease to cirrhosis. Further well-designed studies are needed to corroborate the present observations.

COMMENTS

Background

Hepatitis C virus (HCV) infection is a major health care problem. Recently combined antiviral therapy has been ascertained to improve the non-progressive form of chronic liver disease, eliminating the viral load and reducing fibrosis. However, whether HCV-related-liver cirrhosis characterized by a reduced hepatocellular mass and an increased pressure of portal vein can be cured still remains a challenge.

Research frontiers

The hot spots discussed in the article are the main outcomes of this controlled study, i.e., the assessment of real liver function with a simple and repeatable test (TOSCA) that probes the microsomal enzymatic activity and the evaluation of an Ultrasonographic index of portal hypertension.

Innovations and breakthroughs

The differences from the other related or similar studies relay on the particular schedule of combined antiviral therapy for the treatment of HCV-related liver cirrhosis (low long-protracted doses).

Applications

The perspectives of future application of this research could be expanded by larger and double-blinded studies to validate the good cost/benefit ratio.

Terminology

Total overnight salivary caffeine assessment (TOSCA) is comparable to the well-known caffeine clearance. Briefly, patients were allowed to drink coffee up to late afternoon and after overnight intake-washout, a sample of saliva, was collected in the morning of the next day, roughly centrifuged, frozen and stored until analysis.

Peer review

The manuscript describes that antiviral therapy in patients with hepatitis C-related cirrhosis shows a trend to stabilize or even to ameliorate residual liver function. Although the number of patients was rather small, the study provides evidence for how to treat these patients.

REFERENCES

- 1 Alter MJ. Epidemiology of hepatitis C in the West. *Semin Liver Dis* 1995; **15**: 5-14
- 2 Poynard T, Bedossa P, Opolon P. Natural history of liver fibrosis progression in patients with chronic hepatitis C. The OBSVIRC, METAVIR, CLINIVIR, and DOSVIRC groups. *Lancet* 1997; **349**: 825-832
- 3 Fattovich G, Giustina G, Degos F, Tremolada F, Diodati G, Almasio P, Nevens F, Solinas A, Mura D, Brouwer JT, Thomas H, Njapoum C, Casarin C, Bonetti P, Fuschi P, Basho J, Tocco A, Bhalla A, Galassini R, Noventa F, Schalm SW, Realdi G. Morbidity and mortality in compensated cirrhosis type C: a retrospective follow-up study of 384 patients. *Gastroenterology* 1997; **112**: 463-472
- 4 Niederau C, Lange S, Heintges T, Erhardt A, Buschkamp M, Hürter D, Nawrocki M, Kruska L, Hensel F, Petry W, Häussinger D. Prognosis of chronic hepatitis C: results of a large, prospective cohort study. *Hepatology* 1998; **28**: 1687-1695
- 5 Sangiovanni A, Prati GM, Fasani P, Ronchi G, Romeo R, Manini M, Del Ninno E, Morabito A, Colombo M. The natural history of compensated cirrhosis due to hepatitis C virus: A 17-year cohort study of 214 patients. *Hepatology* 2006; **43**: 1303-1310
- 6 Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; **358**: 958-965
- 7 Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL Jr, Häussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Lin A, Hoffman J, Yu J. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; **347**: 975-982
- 8 Salomon JA, Weinstein MC, Hammitt JK, Goldie SJ. Cost-effectiveness of treatment for chronic hepatitis C infection in an evolving patient population. *JAMA* 2003; **290**: 228-237
- 9 Valla DC, Chevallier M, Marcellin P, Payen JL, Trepo C, Fonck M, Bourliere M, Boucher E, Miquet JP, Parlier D, Lemonnier C, Opolon P. Treatment of hepatitis C virus-related cirrhosis: a randomized, controlled trial of interferon alfa-2b versus no treatment. *Hepatology* 1999; **29**: 1870-1875
- 10 Shepherd J, Brodin HF, Cave CB, Waugh NR, Price A, Gabbay J. Clinical- and cost-effectiveness of pegylated interferon alfa in the treatment of chronic hepatitis C: a systematic review and economic evaluation. *Int J Technol Assess Health Care* 2005; **21**: 47-54
- 11 Iacobellis A, Siciliano M, Perri F, Annicchiarico BE, Leandro G, Caruso N, Accadia L, Bombardieri G, Andriulli A. Peginterferon alfa-2b and ribavirin in patients with hepatitis C virus and decompensated cirrhosis: a controlled study. *J Hepatol* 2007; **46**: 206-212
- 12 Tarantino G, Conca P, Capone D, Gentile A, Polichetti G, Basile V. Reliability of total overnight salivary caffeine assessment (TOSCA) for liver function evaluation in compensated cirrhotic patients. *Eur J Clin Pharmacol* 2006; **62**: 605-612
- 13 Bolognesi M, Sacerdoti D, Merkel C, Gerunda G, Maffei-Faccioli A, Angeli P, Jemolo RM, Bombonato G, Gatta A. Splenic Doppler impedance indices: influence of different portal hemodynamic conditions. *Hepatology* 1996; **23**: 1035-1040
- 14 Giannini E, Fasoli A, Botta F, Testa E, Romagnoli P, Ceppa P, Testa R. Long-term follow up of chronic hepatitis C patients after alpha-interferon treatment: a functional study. *J Gastroenterol Hepatol* 2001; **16**: 399-405
- 15 Schulze-Krebs A, Preimel D, Popov Y, Bartenschlager R, Lohmann V, Pinzani M, Schuppan D. Hepatitis C virus-replicating hepatocytes induce fibrogenic activation of hepatic stellate cells. *Gastroenterology* 2005; **129**: 246-258
- 16 Novo E, Marra F, Zamara E, Valfrè di Bonzo L, Caligiuri A, Cannito S, Antonaci C, Colombatto S, Pinzani M, Parola M. Dose dependent and divergent effects of superoxide anion on cell death, proliferation, and migration of activated human hepatic stellate cells. *Gut* 2006; **55**: 90-97
- 17 Nieto N, Dominguez-Rosales JA, Fontana L, Salazar A, Armendariz-Borunda J, Greenwel P, Rojkind M. Rat hepatic stellate cells contribute to the acute-phase response with increased expression of alpha1 (I) and alpha1 (IV) collagens, tissue inhibitor of metalloproteinase-1, and matrix-metalloproteinase-2 messenger RNAs. *Hepatology* 2001; **33**: 597-607
- 18 Gramenzi A, Andreone P, Fiorino S, Cammà C, Giunta M, Magalotti D, Cursaro C, Calabrese C, Arienti V, Rossi C, Di Febo G, Zoli M, Craxi A, Gasbarrini G, Bernardi M. Impact of interferon therapy on the natural history of hepatitis C virus

related cirrhosis. *Gut* 2001; **48**: 843-848

- 19 **O'Donohue J**, Ng C, Catnach S, Farrant P, Williams R. Diagnostic value of Doppler assessment of the hepatic and portal vessels and ultrasound of the spleen in liver disease. *Eur J Gastroenterol Hepatol* 2004; **16**: 147-155
- 20 **Bayerdörffer E**, Lamerz R, Fliege R, Köpcke W, Mannes GA. Predictive value of serum procollagen-III-peptide for the survival of patients with cirrhosis. *J Hepatol* 1991; **13**: 298-304

S- Editor Liu Y **L- Editor** Wang XL **E- Editor** Yin DH

Secondary pancreatic involvement by a diffuse large B-cell lymphoma presenting as acute pancreatitis

M Wasif Saif, Sapna Khubchandani, Marek Walczak

M Wasif Saif, Yale University School of Medicine, New Haven, CT 06520, United States

Sapna Khubchandani, Marek Walczak, Griffin Hospital, Derby, CT 06520, United States

Correspondence to: M Wasif Saif, MD, MBBS, Associate Professor, Section of Medical Oncology, Yale University School of Medicine, 333 Cedar Street, FMP: 116, New Haven, CT 06520, United States. wasif.saif@yale.edu

Telephone: +1-203-7371569 Fax: +1-203-7853788

Received: May 3, 2007 Revised: June 9, 2007

<http://www.wjgnet.com/1007-9327/13/4909.asp>

Abstract

Diffuse large B-cell lymphoma is the most common type of non-Hodgkin's lymphoma. More than 50% of patients have some site of extra-nodal involvement at diagnosis, including the gastrointestinal tract and bone marrow. However, a diffuse large B-cell lymphoma presenting as acute pancreatitis is rare. A 57-year-old female presented with abdominal pain and matted lymph nodes in her axilla. She was admitted with a diagnosis of acute pancreatitis. Abdominal computed tomography (CT) scan showed diffusely enlarged pancreas due to infiltrative neoplasm and peripancreatic lymphadenopathy. Biopsy of the axillary mass revealed a large B-cell lymphoma. The patient was classified as stage IV, based on the Ann Arbor Classification, and as having a high-risk lymphoma, based on the International Prognostic Index. She was started on chemotherapy with CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone). Within a week after chemotherapy, the patient's abdominal pain resolved. Follow-up CT scan of the abdomen revealed a marked decrease in the size of the pancreas and peripancreatic lymphadenopathy. A literature search revealed only seven cases of primary involvement of the pancreas in B-cell lymphoma presenting as acute pancreatitis. However, only one case of secondary pancreatic involvement by B-cell lymphoma presenting as acute pancreatitis has been published. Our case appears to be the second report of such a manifestation. Both cases responded well to chemotherapy.

© 2007 WJG. All rights reserved.

Key words: Non-Hodgkin's lymphoma; Acute pancreatitis; Pancreatic lymphoma

Saif MW, Khubchandani S, Walczak M. Secondary pancreatic involvement by a diffuse large B-cell lymphoma presenting as acute pancreatitis. *World J Gastroenterol* 2007; 13(36): 4909-4911

INTRODUCTION

Non Hodgkin's lymphoma (NHL) frequently arises in extra-nodal sites, with about 50% of patients having extra-nodal involvement^[1]. The gastrointestinal tract is the most frequent site of involvement, with the stomach and intestines being involved in most cases^[2]. Involvement of the pancreas by NHL has been infrequently reported^[3,4]. Only about 0.2%-2% of patients with NHL have pancreatic involvement at the time of presentation^[1,4,5]. The present report describes a unique case of NHL with secondary pancreatic involvement presenting as acute pancreatitis. A literature search has revealed seven cases of primary involvement of the pancreas by B-cell lymphoma presenting as acute pancreatitis^[6,7]. However, only one case of secondary pancreatic involvement by B-cell lymphoma presenting as acute pancreatitis has been reported^[8].

CASE REPORT

A 57-year-old Caucasian female presented to her primary medical doctor with complaints of abdominal pain. Pain was located in the epigastrium, was dull in character, constant, and grade 3-4/10 in severity. On examination, the patient was also found to have a painful right axillary mass, which appeared to be matted lymph nodes. A biopsy of the matted lymph nodes was undertaken.

However, 4 d later, the patient presented to our emergency department because of worsening abdominal pain. Pain had increased to a severity of 9-10/10 and was associated with nausea and bilious vomiting. On physical examination, she was found to have extreme epigastric tenderness. Laboratory investigations showed an elevated serum amylase level of 623 U/L (normal range 30-110 U/L) and a serum lipase level of 4963 U/L (normal range 23-300 U/L). The patient was initially diagnosed with acute pancreatitis and admitted to the hospital. Past medical history was negative for alcohol consumption or medication use. Abdominal ultrasound done at the time of admission did not reveal any gallstones. Electrolytes and lipid panel were completely normal.

A subsequent abdominal computed tomography (CT) scan showed a diffusely enlarged pancreas due to an infiltrative neoplasm, along with bulky retroperitoneal lymphadenopathy, bulky peripancreatic, mesenteric adenopathy, ascites and peritoneal carcinomatosis

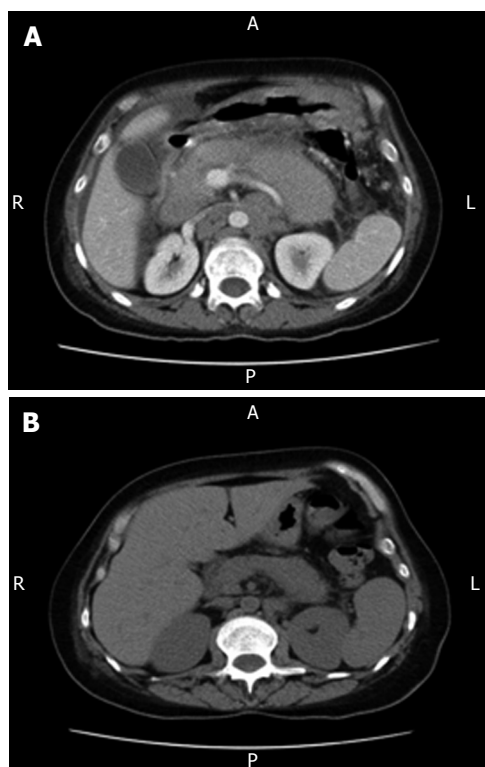


Figure 1 A: Baseline CT scan (pre-therapy). Abdominal CT scan shows diffusely enlarged pancreas due to infiltrative neoplasm, along with bulky retroperitoneal lymphadenopathy, bulky peripancreatic, mesenteric adenopathy, ascites and peritoneal carcinomatosis; B: Post-therapy CT Scan (non-enhanced). A follow-up CT scan of the abdomen reveals a marked decrease in size of the pancreas and retroperitoneal lymph nodes.

(Figure 1A). A report of the biopsy of the axillary mass was available the day after admission, and showed fragments of mononuclear cells with features consistent with lymphocytes, predominantly large in size, with frequent mitotic figures and focal necrosis (Figure 2). Immunohistochemical analysis showed features consistent with a large B-cell lymphoma. Eleven immunohistochemical stains were performed, including CD20, which was positive. Serum lactic dehydrogenase level was elevated to 1227 IU/L (normal range 300-600 U/L) at the time of admission.

Bone marrow biopsy showed evidence of bone marrow involvement by the diffuse large B-cell lymphoma. The patient was classified as stage IV A, based on the Ann Arbor classification designed for Hodgkin's and non-Hodgkin's lymphoma. She was classified as high risk on the International Prognostic Index for diffuse large B-cell lymphoma.

The patient received one cycle of chemotherapy with cyclophosphamide (750 mg/m² Intravenous), doxorubicin (50 mg/m² intravenous), vincristine (1.4 mg/m² intravenous) and prednisone (100 mg/m² orally daily for 5 consecutive days) (CHOP regimen). Within a week after chemotherapy, the patient's abdominal pain had resolved and nausea and vomiting had significantly decreased. Her serum amylase and lipase levels returned to normal (Table 1). A follow-up CT scan of the abdomen revealed a marked decrease in the size of the pancreas and retroperitoneal lymph nodes (Figure 1B).

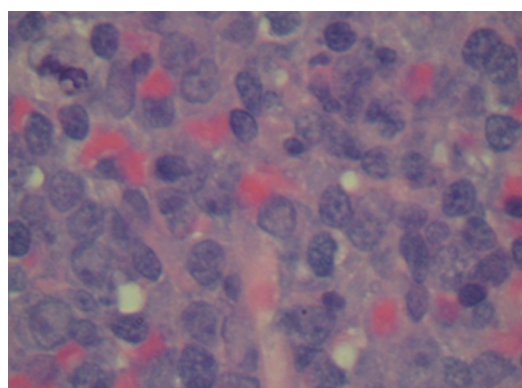


Figure 2 Biopsy of the axillary mass reveals a diffuse large B-cell lymphoma.

Table 1 Differences in serum amylase and lipase levels before and after treatment

	Serum amylase level (U/L)	Serum lipase (U/L)
Before chemotherapy	623	4963
Two days after chemotherapy	72	122
Four days after chemotherapy	45	97

DISCUSSION

Malignant lymphoma infrequently involves the pancreas. The estimated frequency of primary NHL of the pancreas is about 2.2%^[4]. Secondary involvement of the pancreas by NHL is rare. Approximately 0.2%-2% of patients with NHL have pancreatic involvement at presentation^[1,4,5]. An autopsy series of 1269 cases of NHL found pancreatic involvement in 28.9% of the cases^[8]. A literature search of the PubMed database has revealed seven cases of primary involvement of the pancreas by B-cell lymphoma, presenting as acute pancreatitis^[6]. However, only one case of secondary involvement of the pancreas by B-cell lymphoma presenting as acute pancreatitis has been described^[6]. As in our case of secondary pancreatic involvement, that previous patient also presented with acute abdominal pain radiating to the back. He was found to have elevated serum amylase and serum lipase. A CT scan showed diffuse swelling of the pancreas with two masses, one in the corpus and one in the tail. There was also lymphadenopathy near the pancreas. CT of the thorax showed a large lymph node in the mid mediastinum. This lymph node was biopsied and revealed a large B-cell lymphoma. This case of secondary pancreatic involvement also responded well to chemotherapy. A standard regimen of chemotherapy led to normalization of serum lipase and amylase levels, as well as a decrease in the size of the pancreas and the peripancreatic and retroperitoneal lymph nodes.

Diffuse large B-cell lymphoma is the most common NHL, and makes up 30% of newly diagnosed cases. The lymphoma can present with enlarged lymph nodes at either the primary or extra-nodal sites. More than 50% of patients have some extra-nodal involvement at diagnosis^[1]. The most common sites are the gastrointestinal tract and

bone marrow, each being involved in 15%-20% of cases.

The presenting symptoms of pancreatic lymphoma are usually non-specific and include abdominal pain (83%), abdominal mass (58%), weight loss (50%), jaundice (37%), acute pancreatitis (12%), small bowel obstruction (12%) and diarrhea (12%). These symptoms help distinguish pancreatic lymphoma from pancreatic carcinoma^[2]. Imaging plays an important role in the diagnosis and staging of pancreatic masses^[5,9,12]. This is particularly true for pancreatic lymphoma, as treatment and prognosis are significantly different from those for pancreatic adenocarcinoma^[9]. A CT scan is the modality commonly used for the detection of pancreatic lymphoma. Two types of morphological presentation have been reported on CT; one is a tumor-like, localized, well-circumscribed mass presenting as a hypoechogenic mass, and the second is a diffuse enlargement infiltrating the pancreas^[9,10]. The well-circumscribed tumoral form is distinguished from pancreatic adenocarcinoma by the absence of pancreatic duct involvement and the presence of surrounding lymphadenopathy^[9,10]. The imaging findings in the second type are similar to those in acute pancreatitis. Ultrasound- or CT-guided fine needle biopsy of the pancreatic mass can also help distinguish pancreatic lymphoma from pancreatic adenocarcinoma^[4]. In the present case, pancreas biopsy was not necessary to diagnose the lymphoma because sufficient tissue was obtained from the axillary mass.

Anthracycline-based chemotherapy is the standard treatment for NHL, and includes six to eight cycles of R-CHOP for patients of all ages^[11]. High-grade pancreatic lymphoma generally responds well to standard chemotherapy^[11].

Our patient presented with acute abdominal pain and was in the high-risk category of the International Prognostic Index, and was therefore treated as an emergency with high-dose inductive chemotherapy. She responded very well to the chemotherapy and following the first cycle, there was a decrease in the size of the pancreas, as well as the peripancreatic and retroperitoneal lymph nodes. The patient's abdominal pain resolved and her serum amylase and lipase levels normalized.

Secondary involvement of the pancreas by B-cell lymphoma is a rare occurrence. It is important to include such secondary involvement in the differential diagnosis of patients that present with acute pancreatitis. Pathologic

diagnosis is important in distinguishing pancreatic lymphoma from pancreatic carcinoma. Most cases of pancreatic lymphoma respond very well to chemotherapy.

ACKNOWLEDGMENTS

We thank Haswell James E, MD and Soldano Lucille, MD for helping us prepare the manuscript and for their input. A special thanks to the American Cancer Society for their support.

REFERENCES

- 1 **Kiresi DA**, Kivrak AS, Ecirli S, Toy H. Secondary breast, pancreatic, and renal involvement with non-Hodgkin's lymphoma: Imaging findings. *Breast* 2006; **15**: 106-110
- 2 **Saif MW**. Primary pancreatic lymphomas. *JOP* 2006; **7**: 262-273
- 3 **Freeman C**, Berg JW, Cutler SJ. Occurrence and prognosis of extranodal lymphomas. *Cancer* 1972; **29**: 252-260
- 4 **Webb TH**, Lillemoe KD, Pitt HA, Jones RJ, Cameron JL. Pancreatic lymphoma. Is surgery mandatory for diagnosis or treatment? *Ann Surg* 1989; **209**: 25-30
- 5 **Baylor SM**, Berg JW. Cross-classification and survival characteristics of 5,000 cases of cancer of the pancreas. *J Surg Oncol* 1973; **5**: 335-358
- 6 **Bernardeau M**, Auroux J, Cavicchi M, Haioun C, Tsakiris L, Delchier JC. Secondary pancreatic involvement by diffuse large B-cell lymphoma presenting as acute pancreatitis: treatment and outcome. *Pancreatol* 2002; **2**: 427-430
- 7 **Mofredj A**, Cadranet JF, Darchy B, Barbare JC, Cazier A, Pras V, Biour M. Hepatotoxicity caused by therapeutic doses of paracetamol in alcoholics. Report of 2 cases of fatal hepatitis in cirrhosis. *Ann Med Interne (Paris)* 1999; **150**: 507-511
- 8 **Rosenberg SA**, Diamond HD, Craver LF. Lymphosarcoma: the effects of therapy and survival in 1,269 patients in a review of 30 years' experience. *Ann Intern Med* 1960; **53**: 877-897
- 9 **Merkle EM**, Bender GN, Brambs HJ. Imaging findings in pancreatic lymphoma: differential aspects. *AJR Am J Roentgenol* 2000; **174**: 671-675
- 10 **Van Beers B**, Lalonde L, Soyer P, Grandin C, Trigaux JP, De Ronde T, Dive C, Pringot J. Dynamic CT in pancreatic lymphoma. *J Comput Assist Tomogr* 1993; **17**: 94-97
- 11 **Practice guidelines in Oncology-v.2**. 2006: National Comprehensive Cancer Network; Non Hodgkin's lymphoma. Available from: URL: http://www.nccn.org/professionals/physician_gls/PDF/nhl.pdf
- 12 **Bender GN**, Case B, Tsuchida A, Timmons JH, Williard W, Lyons MF, Makuch R. Using sector endoluminal ultrasound to identify the normal pancreas when axial computed tomography is falsely positive. *Invest Radiol* 1999; **34**: 71-74

S- Editor Zhu LH L- Editor Kerr C E- Editor Yin DH

ACKNOWLEDGMENTS

Acknowledgments to Reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those were published and those were rejected in this issue) during the last editing period of time.

Taku Aoki, MD

Division of Hepato-Biliary-Pancreatic and Transplantation Surgery, Department of Surgery, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-8655, Japan

Hitoshi Asakura, Director, Emeritus Professor

International Medical Information Center, Shinanomachi Renga Bldg.35, Shinanomachi, Shinjuku-ku, Tokyo 160-0016, Japan

Marc Basson, MD, PhD, MBA, Chief of Surgery

John D. Dingell VA Medical Center, 4646 John R. Street, Detroit, MI 48301, United States

Luigi Bonavina, Professor

Department of Surgery, Policlinico San Donato, University of Milano, via Morandi 30, Milano 20097, Italy

Christa Buechler, PhD

Regensburg University Medical Center, Internal Medicine I, Franz Josef Strauss Allee 11, 93042 Regensburg, Germany

Giovanni D De Palma, Professor

Department of Surgery and Advanced Technologies, University of Naples Federico II, School of Medicine, Naples 80131, Italy

Mark A Feitelson, Dr

Pathology, Anatomy and Cell Biology of Thomas Jefferson University, Philadelphia 19107, United States

Mitsuhiro Fujishiro, Dr

Department of Gastroenterology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan

Nikolaus Gassler, Professor

Institute of Pathology, University Hospital RWTH Aachen, Pauwelsstrasse 30, 52074 Aachen, Germany

Marek Hartleb

Gastroenterologii, CSK, ul. Medyków 14, 40-752 Katowice, Poland

Yik-Hong Ho, Professor

Department of Surgery, School of Medicine, James Cook University, Townsville 4811, Australia

Kei Ito, MD

Department of Gastroenterology, Sendai City Medical Center, 5-22-1, Tsurugaya, Miyagino-ku, Sendai City 983-0824, Japan

Yoshiaki Iwasaki, Dr

Department of Gastroenterology and Hepatology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, 2-5-1, Shikata-cho, Okayama 700-8558, Japan

Serdar Karakose, Dr, Professor

Department of Radiology, Meram Medical Faculty, Selcuk University, Konya 42080, Turkey

Karlsen Tom Hemming Karlsen, MD

Institute of Immunology, Rikshospitalet University Hospital, N-0027 Oslo, Norway

Andrej Khandoga, MD

Institute for Surgical Research Ludwig-Maximilians-University of Munich, Marchioninistr. 27, 81377 Munich, Germany

Myung-Hwan Kim, Professor

Department of Internal Medicine, University of Ulsan College of Medicine, Asan Medical Center, 388-1 Pungnap-dong, Songpa-gu, Seoul 138-736, South Korea

Leonidas G Koniaris, Professor

Alan Livingstone Chair in Surgical Oncology, 3550 Sylvester Comprehensive Cancer Center (310T), 1475 NW 12th Ave., Miami, FL 33136, United States

James YW Lau

Department of Surgery, Prince of Wales Hospital, the Chinese University of Hong Kong, Hong Kong, China

Masaki Nagaya, MD, PhD

Islet Transplantation and Cell Biology, Joslin Diabetes Center, Room 507, One Joslin Place, Boston, MA 02215, United States

Francesco Negro, MD

Divisions of Gastroenterology and Hepatology and of Clinical Pathology, Hôpital Cantonal Universitaire, 24 rue Micheli-du-Crest, CH-1211 Genève 14, Switzerland

Phillip S Oates, Dr

Department of Physiology, School of Biomedical and Chemical Sciences, The University of Western Australia, Perth, WA, Australia

Johann Ockenga, Dr

Gastroenterology, Hepatology and Endocrinology, Charite-Universitätsmedizin Berlin, Schumannstrasse 20/21, 10117 Berlin, Germany

Jae-Gahb Park, Professor

Seoul National University College of Medicine, 28 Yongon-dong, Chongno-gu, Seoul 110-744, South Korea

Michiie Sakamoto, Professor

Department of Pathology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan

Mitsuo Shimada, Professor

Department of Digestive and Pediatric Surgery, Tokushima University, Kuramoto 3-18-15, Tokushima 770-8503, Japan

Ross C Smith, Professor

Department of Surgery, University of Sydney, Royal North Shore Hospital, St Leonards, New South Wales 2065, Australia

Ulrike Susanne Stein, PhD, Assistant Professor

Max-Delbrück-Center for Molecular Medicine, Robert-Rössle-Straße 10, 13125 Berlin, Germany

Shingo Tsuji, Professor

Department of Internal Medicine and Therapeutics, Osaka University Graduate School of Medicine(A8), 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

Meetings

MAJOR MEETINGS COMING UP

Meeting Falk Research Workshop: Morphogenesis and Cancerogenesis of the Liver
25-26 January 2007
Goettingen
symposia@falkfoundation.de

Meeting Canadian Digestive Diseases Week (CDDW)
16-20 February 2007
Banff-AB
cagoffice@cag-acg.org
www.cag-acg.org/cddw/cddw2007.htm

Meeting Falk Symposium 158: Intestinal Inflammation and Colorectal Cancer
23-24 March 2007
Sevilla
symposia@falkfoundation.de

Meeting BSG Annual Meeting
26-29 March 2007
Glasgow
www.bsg.org.uk/

NEXT 6 MONTHS

Meeting 42nd Annual Meeting of the European Association for the Study of the Liver
11-15 April 2007
Barcelona
easl2007@easl.ch
www.easl.ch/liver-meeting/

Meeting Falk Symposium 159: IBD 2007 - Achievements in Research and Clinical Practice
4-5 May 2007
Istanbul
symposia@falkfoundation.de

Meeting European Society for Paediatric Gastroenterology, Hepatology and Nutrition Congress 2007
9-12 May 2007
Barcelona
espghan2007@colloquium.fr

Digestive Disease Week
19-24 May 2007
Washington Convention Center, Washington DC

Meeting Gastrointestinal Endoscopy Best Practices: Today and Tomorrow, ASGE Annual Postgraduate Course at DDW
23-24 May 2007
Washington-DC
tkoral@asge.org

Meeting ESGAR 2007 18th Annual Meeting and Postgraduate Course
12-15 June 2007
Lisbon
fca@netvisao.pt

Meeting Falk Symposium 160: Pathogenesis and Clinical Practice in

Gastroenterology
15-16 June 2007
Portoroz
symposia@falkfoundation.de

Meeting ILTS 13th Annual International Congress
20-23 June 2007
Rio De Janeiro
www.ils.org

Meeting 9th World Congress on Gastrointestinal Cancer
27-30 June 2007
Barcelona
meetings@imedex.com

EVENTS AND MEETINGS IN 2007

Meeting Falk Research Workshop: Morphogenesis and Cancerogenesis of the Liver
25-26 January 2007
Goettingen
symposia@falkfoundation.de

Meeting Canadian Digestive Diseases Week (CDDW)
16-20 February 2007
Banff-AB
cagoffice@cag-acg.org
www.cag-acg.org/cddw/cddw2007.htm

Meeting Falk Symposium 158: Intestinal Inflammation and Colorectal Cancer
23-24 March 2007
Sevilla
symposia@falkfoundation.de

Meeting BSG Annual Meeting
26-29 March 2007
Glasgow
www.bsg.org.uk/

Meeting 42nd Annual Meeting of the European Association for the Study of the Liver
11-15 April 2007
Barcelona
easl2007@easl.ch
www.easl.ch/liver-meeting/

Meeting Falk Symposium 159: IBD 2007 - Achievements in Research and Clinical Practice
4-5 May 2007
Istanbul
symposia@falkfoundation.de

Meeting European Society for Paediatric Gastroenterology, Hepatology and Nutrition Congress 2007
9-12 May 2007
Barcelona
espghan2007@colloquium.fr

Meeting Gastrointestinal Endoscopy Best Practices: Today and Tomorrow, ASGE Annual Postgraduate Course at DDW
23-24 May 2007
Washington-DC
tkoral@asge.org

Meeting ESGAR 2007 18th Annual Meeting and Postgraduate Course
12-15 June 2007
Lisbon
fca@netvisao.pt

Meeting Falk Symposium 160: Pathogenesis and Clinical Practice in Gastroenterology
15-16 June 2007
Portoroz
symposia@falkfoundation.de

Meeting ILTS 13th Annual International Congress
20-23 June 2007
Rio De Janeiro
www.ils.org

Meeting 9th World Congress on Gastrointestinal Cancer
27-30 June 2007
Barcelona
meetings@imedex.com

Meeting 15th International Congress of the European Association for Endoscopic Surgery
4-7 July 2007
Athens
info@eaes-eur.org
congresses.eaes-eur.org/

Meeting 39th Meeting of the European Pancreatic Club
4-7 July 2007
Newcastle
www.e-p-c2007.com

Meeting XXth International Workshop on Helicobacter and related bacteria in cronic degistive inflammation
20-22 September 2007
Istanbul
www.heliobacter.org

Meeting Falk Workshop: Mechanisms of Intestinal Inflammation
10 October 2007
Dresden
symposia@falkfoundation.de

Meeting Falk Symposium 161: Future Perspectives in Gastroenterology
11-12 October 2007
Dresden
symposia@falkfoundation.de

Meeting Falk Symposium 162: Liver Cirrhosis - From Pathophysiology to Disease Management
13-14 October 2007
Dresden
symposia@falkfoundation.de

American College of Gastroenterology Annual Scientific Meeting
12-17 October 2007
Pennsylvania Convention Center Philadelphia, PA

Meeting APDW 2007 - Asian Pacific Digestive Disease Week 2007
15-18 October 2007
Kobe
apdw@convention.co.jp
www.apdw2007.org

15th United European Gastroenterology Week, UEGW
27-31 October 2007
Le Palais des Congrès de Paris, Paris, France

Meeting The Liver Meeting® 2007 - 57th Annual Meeting of the American Association for the Study of Liver Diseases

2-6 November 2007
Boston-MA
www.aasld.org

Gastro 2009, World Congress of Gastroenterology and Endoscopy London, United Kingdom 2009

Instructions to authors

GENERAL INFORMATION

World Journal of Gastroenterology (WJG, *World J Gastroenterol* ISSN 1007-9327 CN 14-1219/R) is a weekly journal of more than 48 000 circulation, published on the 7th, 14th, 21st and 28th of every month.

Original Research, Clinical Trials, Reviews, Comments, and Case Reports in esophageal cancer, gastric cancer, colon cancer, liver cancer, viral liver diseases, etc., from all over the world are welcome on the condition that they have not been published previously and have not been submitted simultaneously elsewhere.

Indexed and abstracted in

Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®) and Journal Citation Reports/Science Edition, *Index Medicus*, MEDLINE and PubMed, Chemical Abstracts, EMBASE/Excerpta Medica, Abstracts Journals, *Nature Clinical Practice Gastroenterology and Hepatology*, CAB Abstracts and Global Health. ISI JCR 2003-2000 IF: 3.318, 2.532, 1.445 and 0.993.

Published by WJG

SUBMISSION OF MANUSCRIPTS

Manuscripts should be typed double-spaced on A4 (297 mm × 210 mm) white paper with outer margins of 2.5 cm. Number all pages consecutively, and start each of the following sections on a new page: Title Page, Abstract, Introduction, Materials and Methods, Results, Discussion, acknowledgements, References, Tables, Figures and Figure Legends. Neither the editors nor the Publisher is responsible for the opinions expressed by contributors. Manuscripts formally accepted for publication become the permanent property of WJG, and may not be reproduced by any means, in whole or in part without the written permission of both the authors and the Publisher. We reserve the right to put onto our website and copy-edit accepted manuscripts. Authors should also follow the guidelines for the care and use of laboratory animals of their institution or national animal welfare committee.

Authors should retain one copy of the text, tables, photographs and illustrations, as rejected manuscripts will not be returned to the author(s) and the editors will not be responsible for the loss or damage to photographs and illustrations in mailing process.

Online submission

Online submission is strongly advised. Manuscripts should be submitted through the Online Submission System at: <http://www.wjgnet.com/index.jsp>. Authors are highly recommended to consult the ONLINE INSTRUCTIONS TO AUTHORS (<http://www.wjgnet.com/wjg/help/instructions.jsp>) before attempting to submit online. Authors encountering problems with the Online Submission System may send an email you describing the problem to wjg@wjgnet.com for assistance. If you submit your manuscript online, do not make a postal contribution. A repeated online submission for the same manuscript is strictly prohibited.

Postal submission

Send 3 duplicate hard copies of the full-text manuscript typed double-spaced on A4 (297 mm × 210 mm) white paper together with any original photographs or illustrations and a 3.5 inch computer diskette or CD-ROM containing an electronic copy of the manuscript including all the figures, graphs and tables in native Microsoft Word format or *.rtf format to:

Editorial Office

World Journal of Gastroenterology

Editorial Department: Apartment 1066, Yishou Garden,
58 North Langxinzhuang Road,
PO Box 2345, Beijing 100023, China
E-mail: wjg@wjgnet.com
<http://www.wjgnet.com>
Telephone: +86-10-85381892
Fax: +86-10-85381893

MANUSCRIPT PREPARATION

All contributions should be written in English. All articles must be submitted using a word-processing software. All submissions must be typed in 1.5

line spacing and in word size 12 with ample margins. The letter font is Tahoma. For authors from China, one copy of the Chinese translation of the manuscript is also required (excluding references). Style should conform to our house format. Required information for each of the manuscript sections is as follows:

Title page

Full manuscript title, running title, all author(s) name(s), affiliations, institution(s) and/or department(s) where the work was accomplished, disclosure of any financial support for the research, and the name, full address, telephone and fax numbers and email address of the corresponding author should be included. Titles should be concise and informative (removing all unnecessary words), emphasize what is new, and avoid abbreviations. A short running title of less than 40 letters should be provided. List the author(s)' name(s) as follows: initial and/or first name, middle name or initial(s) and full family name.

Abstract

An informative, structured abstract of no more than 350 words should accompany each manuscript. Abstracts for original contributions should be structured into the following sections: AIM: Only the purpose should be included. METHODS: The materials, techniques, instruments and equipments, and the experimental procedures should be included. RESULTS: The observatory and experimental results, including data, effects, outcome, etc. should be included. Authors should present *P* value where necessary, and the significant data should accompany. CONCLUSION: Accurate view and the value of the results should be included.

The format of structured abstracts is at: <http://www.wjgnet.com/wjg/help/11.doc>

Key words

Please list 5-10 key words that could reflect content of the study mainly from *Index Medicus*.

Text

For most article types, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include in appropriate Figures and Tables. Data should be presented in the body text or in Figures and Tables, but not in both.

Illustrations

Figures should be numbered as 1, 2, 3 and so on, and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. No detailed legend should be involved under the figures. This part should be added into the text where the figures are applicable. Digital images: black and white photographs should be scanned and saved in TIFF format at a resolution of 300 dpi; color images should be saved as CMYK (print files) but not as RGB (screen-viewing files). Place each photograph in a separate file. Print images: supply images of size no smaller than 126 mm × 85 mm printed on smooth surface paper; label the image by writing the Figure number and orientation using an arrow. Photomicrographs: indicate the original magnification and stain in the legend. Digital Drawings: supply files in EPS if created by freehand and illustrator, or TIFF from photoshops. EPS files must be accompanied by a version in native file format for editing purposes. Existing line drawings should be scanned at a resolution of 1200 dpi and as close as possible to the size where they will appear when printed. Please use uniform legends for the same subjects. For example: Figure 1 Pathological changes of atrophic gastritis after treatment. A: ...; B: ...; C: ...; D: ...; E: ...; F: ...; G: ...

Tables

Three-line tables should be numbered as 1, 2, 3 and so on, and mentioned clearly in the main text. Provide a brief title for each table. No detailed legend should be included under the tables. This part should be added into the text where the tables are applicable. The information should complement but not duplicate that contained in the text. Use one horizontal line under the title, a second under the column heads, and a third below the Table, above any footnotes. Vertical and italic lines should be omitted.

Notes in tables and illustrations

Data that are not statistically significant should not be noted. ^a*P*<0.05, ^b*P*<0.01 should be noted (*P*>0.05 should not be noted). If there are other series of *P* values, ^c*P*<0.05 and ^d*P*<0.01 are used. Third series of *P* values can be expressed as ^e*P*<0.05 and ^f*P*<0.01. Other notes in tables or under

illustrations should be expressed as 1F , 2F , 3F ; or some other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, etc. in a certain sequence.

Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscripts and who endorse the data and conclusions are included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

REFERENCES

Coding system

The author should code the references according the citation order in text in Arabic numerals, put references codes in square brackets, superscript it at the end of citation content or the author name of the citation. For those citation content as the narrate part, the coding number and square brackets should be typeset normally. For example, Crohn's disease (CD) is associated with increased intestinal permeability^[1,2]. If references are directly cited in the text, they would be put together with the text, for example, from references [19,22-24], we know that...

When the authors code the references, please ensure that the order in text is the same as in reference part and also insure the spelling accuracy of the first author's name. Do not code the same citation twice.

PMID requirement

PMID roots in the abstract serial number indexed by PubMed (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed>). The author should supply the PMID for journal citation. For those references that have not been indexed by PubMed, a printed copy of the first page of the full reference should be submitted.

The accuracy of the information of the journal citations is very important. Through reference testing system, the authors and editor could check the authors name, title, journal title, publication date, volume number, start page, and end page. We will interlink all references with PubMed in ASP file so that the readers can read the abstract of the citations online immediately.

Style for journal references

Authors: the first author should be typed in bold-faced letter. The surname of all authors should be typed with the initial letter capitalized and followed by their name in abbreviation (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR). Title of the cited article and italicized journal title (Journal title should be in its abbreviation form as shown in PubMed), publication date, volume number (in black), start page, and end page [PMID: 11819634]

Note: The author should test the references through reference testing system (<http://www.wjgnet.com/cgi-bin/index.pl>)

Style for book references

Authors: the first author should be typed in bold-faced letter. The surname of all authors should be typed with the initial letter capitalized and followed by their name in abbreviation (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR) Book title. Publication number. Publication place: Publication press, Year: start page and end page.

Format

Journals

English journal article (list all authors and include the PMID where applicable)

- 1 **Grover VP**, Dresner MA, Forton DM, Counsell S, Larkman DJ, Patel N, Thomas HC, Taylor-Robinson SD. Current and future applications of magnetic resonance imaging and spectroscopy of the brain in hepatic encephalopathy. *World J Gastroenterol* 2006; **12**: 2969-2978 [PMID: 16718775]

Chinese journal article (list all authors and include the PMID where applicable)

- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci U S A* 2006; In press

Organization as author

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

Both personal authors and an organization as author

- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

No author given

- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

Volume with supplement

- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325]

Issue with no volume

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; **(401)**: 230-238 [PMID: 12151900]

No volume or issue

- 9 Outreach: bringing HIV-positive individuals into care. *HRS/A Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ Cell Tumour Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

Patent (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Inappropriate references

Authors should always cite references that are relevant to their article, and avoid any inappropriate references. Inappropriate references include those that are linked with a hyphen and the difference between the two numbers at two sides of the hyphen is more than 5. For example, [1-6], [2-14] and [1, 3, 4-10, 22] are all considered as inappropriate references. Authors should not cite their own unrelated published articles.

Statistical data

Present as mean \pm SD or mean \pm SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as γ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, *m* (B) = 78 kg; blood pressure, *p*(B) = 16.2/12.3 kPa; incubation time, *t* (incubation) = 96 h, blood glucose concentration, *c* (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, *p* (CEA) = 8.6 $24.5 \mu\text{g/L}$; CO₂ volume fraction, 50 mL/L CO₂ not 5% CO₂; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, etc. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

The format about how to accurately write common units and quantum is at: <http://www.wjgnet.com/wjg/help/15.doc>

Abbreviations

Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further mention.

Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gyrA*, *arg 1*, *c myc*, *c fos*, etc.

Restriction enzymes: *EcoRI*, *HindI*, *BamHI*, *Kbo I*, *Kpn I*, etc.

Biology: *H pylori*, *E coli*, etc.

SUBMISSION OF THE REVISED MANUSCRIPTS AFTER ACCEPTED

Please revise your article according to the revision policies of *WJG*. The revised version including manuscript and high-resolution image figures (if any) should be copied on a floppy or compact disk. Author should send the revised manuscript, along with printed high-resolution color or black and white photos, copyright transfer letter, the final check list for authors, and responses to reviewers by a courier (such as EMS) (submission of revised manuscript by e-mail or on the *WJG* Editorial Office Online System is NOT available at present).

Language evaluation

The language of a manuscript will be graded before sending for revision.

(1) Grade A: priority publishing; (2) Grade B: minor language polishing; (3) Grade C: a great deal of language polishing; (4) Grade D: rejected. The revised articles should be in grade B or grade A.

Copyright assignment form

Please download CAF from <http://www.wjgnet.com/wjg/help/9.doc>.

We certify that the material contained in this manuscript:

Ms:

Title:

is original, except when appropriately referenced to other sources, and that written permission has been granted by any existing copyright holders. We agree to transfer to *WJG* all rights of our manuscript, including: (1) all copyright ownership in all print and electronic formats; (2) the right to grant permission to republish or reprint the stated material in whole or in part, with or without a fee; (3) the right to print copies for free distribution or sale; (4) the right to republish the stated material in a collection of articles or in any other format. We also agree that our article be put on the Internet.

Criteria for authorship: The *WJG* requests and publishes information about contributions of each author named to the submitted study. Authorship credit should be based on (1) direct participation in the study, including substantial contributions to conception and design of study, or acquisition of data, or analysis and interpretation of data; (2) manuscript writing, including drafting the article, or revising it critically for important intellectual content; (3) supportive work, including statistical analysis of data, or acquisition of funding, or administration, technology and materials support, or supervision, or supportive contributions. Authors should meet at least one of the three conditions. The *WJG* does not publish co-first authors and co-corresponding authors.

We hereby assign copyright transfer to *WJG* if this paper is accepted.

Author Name in full (Full names should be provided, with first name first, followed by middle names and family name at the last, eg, Eamonn MM Quigley). Handwritten names are not accepted.

Author Name in abbreviation (Family name is put first in full, followed by middle names and first name in abbreviation with first letter in capital, eg, Quigley EMM). Handwritten names are not accepted.

Final check list for authors

The format is at: <http://www.wjgnet.com/wjg/help/13.doc>

Responses to reviewers

Please revise your article according to the comments/suggestions of reviewers. The format for responses to the reviewers' comments is at: <http://www.wjgnet.com/wjg/help/10.doc>

1 Full Name: _____

Abbreviation Name: _____

Signed: _____

Date: _____

2 Full Name: _____

Abbreviation Name: _____

Signed: _____

Date: _____

3 Full Name: _____

Abbreviation Name: _____

Signed: _____

Date: _____

4 Full Name: _____

Abbreviation Name: _____

Signed: _____

Date: _____

5 Full Name: _____

Abbreviation Name: _____

Signed: _____

Date: _____

6 Full Name: _____

Abbreviation Name: _____

Signed: _____

Date: _____

7 Full Name: _____

Abbreviation Name: _____

Signed: _____

Date: _____

8 Full Name: _____

Abbreviation Name: _____

Signed: _____

Date: _____

9 Full Name: _____

Abbreviation Name: _____

Signed: _____

Date: _____

10 Full Name: _____

Abbreviation Name: _____

Signed: _____

Date: _____

Proof of financial support

For paper supported by a foundation, authors should provide a copy of the document and serial number of the foundation.

Publication fee

Authors of accepted articles must pay publication fee.

EDITORIAL and LETTERS TO THE EDITOR are free of charge.