Liver infiltrating T lymphocytes express interferon γ and inducible nitric oxide synthase in chronic hepatitis C virus infection

S Schweyer, S Mihm, H J Radzun, H Hartmann, A Fayyazi

Abstract

***Background*—Pathogenesis of hepatitis C virus (HCV) associated liver injury is thought to be due to the host antiviral immune response. Using a quantitative, competitive RT-PCR technique, we recently showed that expression of interferon γ (IFN-γ) and IFN-γ inducible type of nitric oxide synthase (iNOS) is increased in homogenised liver tissue of patients with chronic HCV infection.**

***Aims*—To determine the cellular origin of IFN-γ and iNOS expression and to examine the hypothesis that T cell derived IFN-γ secretion induces iNOS in hepatocytes in chronic HCV infection.**

***Methods*—By applying a non-radioactive in situ hybridisation method combined with indirect immunofluorescence, 33 liver biopsy specimens from patients with chronic HCV infection were studied for cellular expression of IFN-γ and iNOS mRNA.**

***Results*—In chronic HCV infection, both IFN-γ and iNOS gene expression were significantly increased. IFN-γ and iNOS mRNA were observed in CD3+ lymphocytes infiltrating portal tracts and hepatic lobules, but not in hepatocytes.**

***Conclusions*—Results are consistent with previous reports that IFN-γ and iNOS transcripts are elevated in chronic HCV infection. In contrast to the hypothesis, IFN-γ expressing T cells do not induce iNOS in hepatocytes, but probably in T cells. T lymphocytes expressing IFN-γ and/or iNOS have the potential to participate in autocrine and paracrine pathways that may contribute to the pathobiology of chronic hepatitis C.**

(*Gut* 2000;**46**:255–259)

Keywords: hepatitis C; interferon type II; nitric oxide synthase; in situ hybridisation

Department of Pathology, Division of Pathology, Georg-August University, Göttingen, Germany
S Schweyer
H J Radzun
A Fayyazi

Department of Internal Medicine, Division of Gastroenterology and Endocrinology
S Mihm
H Hartmann

Correspondence to:
Dr S Schweyer, Universitätsklinikum Göttingen, Abteilung Pathologie, Robert-Koch-Strasse 40, D-37075 Göttingen, Germany.

Accepted for publication 18 August 1999

Hepatitis C virus (HCV) is responsible for over 90% of what was previously called non-A non-B hepatitis, most cases of post-transfusion hepatitis, and many sporadic infections.[1] Although more than 50% of infected patients develop a chronic hepatitis,[2] little is known about the HCV associated mechanism(s) leading to liver destruction.[3] We and others have shown that the immune cells and mediators involved in the process of virus elimination are responsible for hepatocellular injury.[4–7] In this respect, the secretion of the T helper 1 (Th1) cytokine, interferon γ (IFN-γ), has been suggested to promote the hepatic pathology.[5–7] Animal models of experimentally induced hepatitis support this hypothesis—for example, in a model of transgenic mice expressing IFN-γ under the control of a liver specific promoter, the animals developed a chronic hepatitis.[8] Furthermore, IFN-γ has been shown to be the principal mediator of the hepatic inflammatory process induced by syngeneic lymphocytes against hepatitis B surface antigen (HBsAg) expressed in the liver of transgenic mice.[9] However, the mechanism of IFN-γ associated liver injury is still unclear.

IFN-γ has been shown to upregulate the inducible isoform of nitric oxide synthase (iNOS) in monocytes/macrophages, resulting in NO production.[10] As a gaseous free radical, monocyte/macrophage derived NO may destroy infected as well as non-infected host cells.[11] Moreover, it is known that stimulated hepatocytes can express iNOS.[12] On the assumption that an IFN-γ dependent induction of iNOS in hepatocytes results in NO production which plays a dual proinflammatory and antiviral role in hepatitis C, we analysed liver biopsy specimens of patients chronically infected with HCV. One part of each biopsy was fixed in formaldehyde and embedded in paraffin wax for histopathological evaluation. The other part was homogenised and analysed for the expression of IFN-γ and iNOS by a quantitative, competitive reverse transcription polymerase chain reaction (RT-PCR).[13] Although it could be shown that in HCV infected patients hepatic iNOS expression is upregulated in an IFN-γ dependent manner, the level of transcription of iNOS did not correlate to liver injury, but correlated to hepatic HCV RNA content. This new evidence was not consistent with our assumption and raised the question whether iNOS is really produced in hepatocytes. To map the cellular origin of IFN-γ and iNOS mRNA, the formalin fixed, paraffin wax embedded counterpart of liver biopsy specimens of the same HCV infected patients were studied by non-radioactive in situ hybridisation combined with immunofluorescence.

Abbreviations used in this paper: ALT, alanine aminotransferase; DIH, drug induced hepatitis; HbsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; IFN-γ, interferon γ; iNOS, inducible nitric oxide synthase; ISH, in situ hybridisation; PBC, primary biliary cirrhosis; Th1, T helper 1.

Materials and methods

PATIENTS, TISSUE SAMPLES, AND HISTOPATHOLOGICAL EVALUATION

Thirty three liver biopsy specimens including three explanted livers from patients with chronic HCV infection were studied (17 women, 16 men; aged 33–65 years, mean 45.2). Chronic HCV infection was diagnosed histopathologically according to established criteria[14] as well as by the presence of anti-HCV antibodies and HCV RNA in sera and/or by elevated serum aspartate aminotransferase and alanine aminotransferase (ALT) activities observed over a period longer than six months. Serum aminotransferase activities ranged from 10 to 108 U/l (mean 43 U/l) for aspartate aminotransferase, and from 12 to 250 U/l (mean 81 U/l) for ALT.

Liver biopsy specimens from patients with chronic hepatitis B (HBV; n=3), primary biliary cirrhosis (PBC; n=1), drug induced hepatitis (DIH; n=2), or without any hepatic disorder (n=10) served as controls. Biopsy specimens without pathological changes were obtained from patients with malignant lymphoma, who underwent liver biopsy as a part of lymphoma staging.

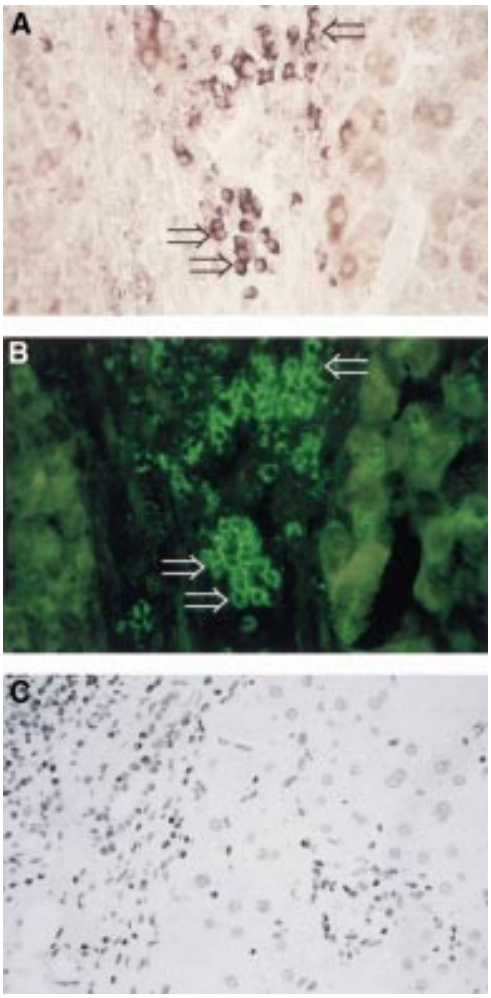

Figure 1 Non-radioactive in situ hybridisation for the antisense cRNA probe of IFN-γ on liver specimens from patients chronically infected with HCV: (A) combined with immunofluoresecent labelling for T cells (CD3); (B) on the same section. Open arrows denote IFN-γ mRNA expressing CD3+ T cells. In situ hybridisation carried out by applying labelled sense cRNA probe of IFN-γ was negative (C). Original magnification × 400.

Tissue samples were fixed in 4% formaldehyde and embedded in paraffin wax. Sections (5–10 μm thick) were mounted on slides coated with 2% 3-aminopropyltriethoxy-silane (Sigma, Munich, Germany) dissolved in acetone. After deparaffinisation, sections were stained histochemically (haematoxylin + eosin, and trichrome), and by applying in situ hybridisation combined with indirect immunofluorescence.

A modified form of the Histology Activity Index designed by Knodell and colleagues[15] served to assess grading and staging of chronic HCV infection, as described previously.[16] In liver biopsy specimens, the prevalence of necroinflammatory changes was graded as mild in 40% (n=13), moderate in 45% (n=15), and severe hepatitis in 15% (n=5) of patients. Architectural alterations (staging) were graded as absent or mild fibrosis in 46% (n=15), moderate fibrosis in 27% (n=9), and notable fibrosis/cirrhosis in 27% (n=9) of patients.

Histopathological evaluation was performed without knowledge of the biochemical or clinical data.

NON-RADIOACTIVE IN SITU HYBRIDISATION

For preparation of riboprobes, cDNA fragments derived from the 3' terminal region of the human IFN-γ (position 381–600 of the coding DNA) and hepatic iNOS gene (position 2406–2658 of the coding DNA) were subcloned into pBluescript II KS+ (Stratagene, California, USA). The subcloned fragments served as templates for in vitro transcription of digoxigenin 11-dUTP labelled antisense and sense probes which were produced by virtue of T3 or T7 RNA polymerase according to the manufacturer's instructions (Boehringer, Mannheim, Germany). In situ hybridisation (ISH) was then performed on deparaffinised sections as described by Breitschopf *et al*.[17] Briefly, sections were digested with proteinase K (10 μg/ml) and incubated overnight with digoxigenin labelled riboprobes at 55°C. Slides were then washed in 1% sodium dodecyl sulphate (SDS) in 2× saline sodium citrate (SSC) (15 minutes at 55°C), and 1% SDS in 1× SSC (20 minutes at room temperature). Finally, they were washed in Tris buffered saline (TBS; 50 mM Tris-HCl, 150 mM NaCl, pH 7,5) containing 0.1% (vol/vol) Tween 20 (Boehringer) and 1% fetal calf serum (CC Pro, Neustadt, Germany), and incubated (two hours at room temperature) with a sheep alkaline phosphatase conjugated polyclonal antibody $F(ab)_2$ fragment against digoxigenin (Boehringer).

Signal was detected using 5-bromo-4-chloro-3-indolyl phosphate as a substrate and nitro blue tetrazolium as a coupler (Boehringer). Liver biopsy specimens were counterstained with Mayer's haematoxylin and mounted in Aquamount.

Samples were then examined by using light microscopy. Positive cells showed strong cytoplasmic staining around the clearly demarcated nuclei. Liver sections from PBC served as controls for ISH experiments with IFN-γ cRNA probes, and skin biopsy specimens from

normal skin and psoriatic lesions for ISH experiments with iNOS cRNA probes.[18 19] Each tissue sample was also stained with equivalent quantities of labelled sense riboprobes.

To evaluate ISH data, sections were studied by two pathologists. The number of labelled infiltrating cells was determined per section in four high power visual fields (original magnification × 400) of portal tracts and hepatic lobules.

INDIRECT IMMUNOFLUORESCENCE

The polyclonal antibody against CD3, and the monoclonal antibody against CD20 (clone L26) were obtained from Dako (Hamburg, Germany). The monoclonal antibody CD68 (clone Ki-M1P) recognising all subpopulations of monocytes/macrophages was kindly provided by the Department of Pathology, University of Kiel, Germany.[20] The primary antibodies were applied at a working dilution of: CD3, 1/50; CD20, 1/20; CD68, 1/2000.

Indirect immunofluorescence was performed immediately after ISH. Sections were incubated for two hours with the primary antibodies. The samples were then incubated with a FITC labelled goat antimouse IgG (working dilution 1/50) for one hour (Dako, Hamburg, Germany). Finally, they were mounted with Fluorescent Mounting Medium (Dako, Hamburg, Germany) and examined using fluorescence microscopy.

For controls, sections from all samples were stained using the above procedures, but omitting the primary or secondary antibodies.

STATISTICS

The non-parametric Mann-Whitney U test and Spearman rank correlation test were applied for the comparison of mean values and for calculation of correlation coefficients, respectively. A value of $p<0.01$ was considered to indicate statistically significant differences.

Results

CELLULAR LOCALISATION OF HEPATIC IFN-γ EXPRESSION

In liver specimens from HCV infected patients, the number of IFN-γ mRNA expressing cells (mean (SD) per 4× high power field) was 35.4 (8.54). IFN-γ mRNA was found to be restricted to mononuclear cells infiltrating portal tracts and hepatic lobules. These cells were characterised as T cells by applying indirect immunofluorescence for CD3 antigen (fig 1A, B). No specific signal could be detected in control samples hybridised with the sense IFN-γ cRNA probe (fig 1C).

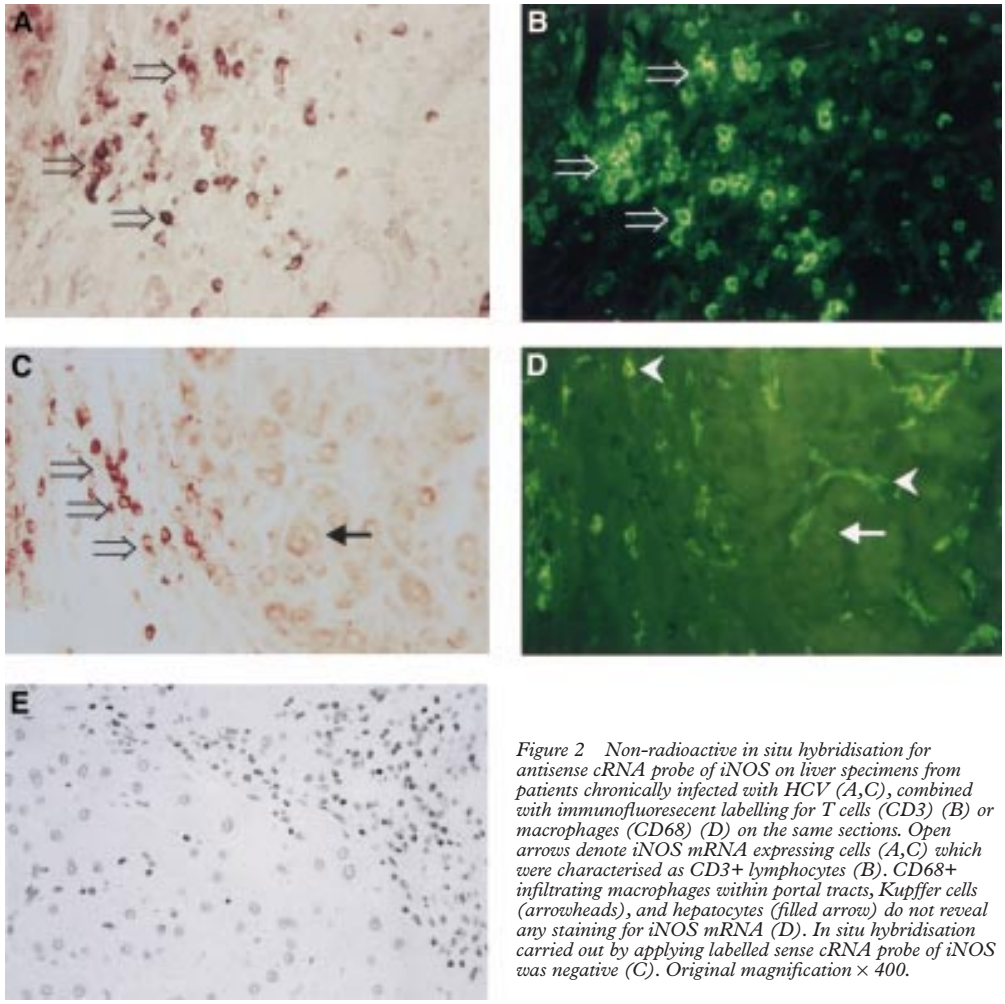

Figure 2 Non-radioactive in situ hybridisation for antisense cRNA probe of iNOS on liver specimens from patients chronically infected with HCV (A,C), combined with immunofluoresecent labelling for T cells (CD3) (B) or macrophages (CD68) (D) on the same sections. Open arrows denote iNOS mRNA expressing cells (A,C) which were characterised as CD3+ lymphocytes (B). CD68+ infiltrating macrophages within portal tracts, Kupffer cells (arrowheads), and hepatocytes (filled arrow) do not reveal any staining for iNOS mRNA (D). In situ hybridisation carried out by applying labelled sense cRNA probe of iNOS was negative (C). Original magnification × 400.

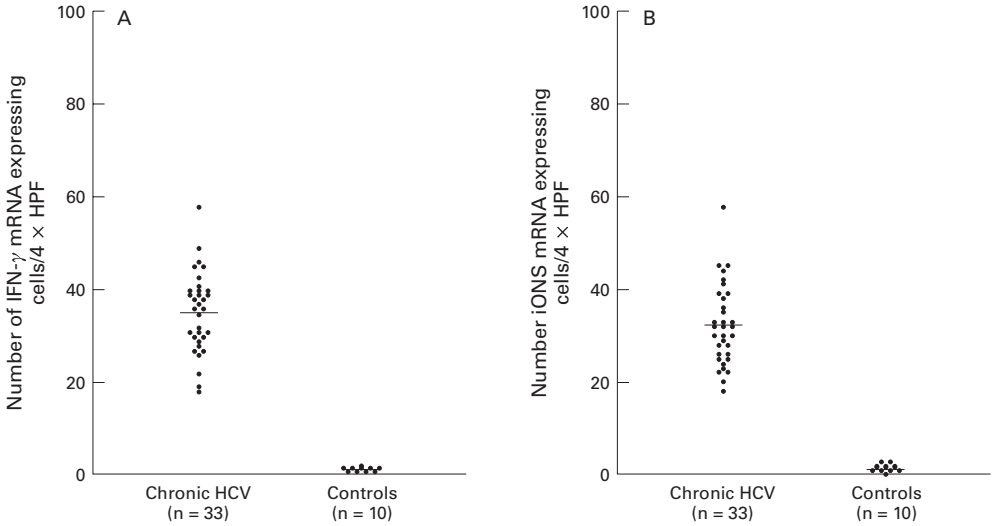

Figure 3 Liver biopsy specimens from patients with chronic HCV infection (n=33) and individuals without any hepatic disorder (n=10) were studied for IFN-γ and iNOS mRNA by applying non-radioactive in situ hybridisation.

In specimens from HBV infected IFN-γ mRNA was found in 36 (5) mononuclear cells, and in those from patients with PBC in 55 mononuclear cells; 4 (1) mononuclear cells in cases of drug induced hepatitis and 0.7 (0.6) mononuclear cells in specimens without a hepatic disorder were positively stained.

The expression of IFN-γ was significantly augmented in specimens from HCV infected patients, compared with liver specimens without a pathological disorder ($p<0.0001$, Mann-Whitney U test; fig 3). The number of IFN-γ expressing cells within the hepatic infiltrate was not correlated to sex ($p=0.7534$, $r=0.0867$, Spearman rank correlation), age ($p=0.4126$, $r=0.1672$), serum ALT activity ($p=0.1880$, $r=0.2633$), or to the degree of fibrosis ($p=0.0544$, $r=0.3846$) (data not shown).

CELLULAR LOCALISATION OF HEPATIC iNOS EXPRESSION

In liver specimens from chronic HCV infected patients the count of iNOS mRNA positive cells was 32.1 (8.2). These cells were characterised as CD3+ T cells infiltrating portal tracts and hepatic lobules (fig 2A, B). By contrast, Ki-M1P+ infiltrating or sessile (Kupffer cells) macrophages, hepatocytes (fig 2C, D), CD20+ B lymphocytes, or vascular endothelium did not reveal any iNOS mRNA expression. No specific signal could be detected in control samples hybridised with the sense iNOS cRNA probe (fig 2E).

In specimens from HBV infected patients iNOS mRNA was found in 39 (4) mononuclear cells and in those from patients with PBC in 58 mononuclear cells, whereas 3.5 (0.5) mononuclear cells in cases of drug induced hepatitis and 1.6 (0.6) mononuclear cells in specimens without a hepatic disorder were stained positively for iNOS mRNA.

The expression of iNOS was found to be significantly augmented in specimens from HCV infected patients, compared with liver specimens without a pathological disorder ($p<0.0001$, Mann-Whitney U test; fig 3). The number of iNOS expressing cells within the hepatic infiltrate was not correlated to sex ($p=0.2780$, $r=0.2993$, Spearman rank correlation), age ($p=0.8559$, $r=0.0371$), serum ALT activity ($p=0.453$, $r=0.1499$), or to the degree of fibrosis ($p=0.4504$, $r=0.1509$) (data not shown).

Discussion

In this study high numbers of IFN-γ and iNOS expressing mononuclear cells were detected in chronic HCV infection. Both IFN-γ and iNOS transcripts were restricted to CD3+ T lymphocytes infiltrating portal tracts and hepatic lobules. In cases of chronic HBV infection and PBC similar amounts of IFN-γ and iNOS transcripts were shown in mononuclear cells, whereas only a few IFN-γ and iNOS expressing mononuclear cells were found in DIH and in liver specimens without any pathological disorder. These results indicate that IFN-γ and iNOS may be involved in T cell mediated hepatic injuries such as viral hepatitis and PBC, but not in those caused by drugs or toxins.

Several studies have pointed to the role of IFN-γ in hepatic pathology in general,[8 9] and in the development of HCV associated hepatocellular injury in particular.[5–7 21] In an autocrine manner, IFN-γ has been shown to act as a growth factor for the proliferation and differentiation of helper and cytotoxic T lymphocytes[22 23] which attack and lyse hepatocytes presenting viral peptides.[24] Nevertheless, in the majority of cases T lymphocytes fail to eradicate viruses harboured in hepatocytes. Previously we reported that in such cases hepatic iNOS expression rises in positive correlation to HCV RNA content.[13] Hepatocytes, macrophages, and B lymphocytes have been shown to express iNOS following stimulation with bacterial products (for example, lipopolysaccharide), cytokines (for example, IFN-γ), or after viral infections (for example, Epstein-Barr virus).[12 25 26] In situ evidence for the expression of iNOS mRNA in liver biopsy specimens from HBV and HCV infected patients was recently published by Majano *et*

al.[27] By applying ISH these authors showed that hepatocytes, vascular endothelium, and infiltrating mononuclear cells express iNOS transcripts.

It is difficult to reconcile published data with our results showing that in liver biopsy specimens from HCV infected patients neither hepatocytes nor endothelial cells express iNOS mRNA. It may be speculated that the shorter iNOS cRNA probe (252 bases instead of 700 bases) and the higher hybridisation temperature (55°C instead of 45°C) used in the present work produced more stringent hybridisation conditions.[28] Subsequently it is possible that such stringency helped to differentiate mRNAs of highly homologous proteins, thereby not producing signals within hepatocytes and endothelial cells, but showing iNOS mRNA in CD3+ T cells.

Many reports have shown that activated T cells express iNOS.[29–31] The subsequent NO production has been shown to: inhibit Th1 cell proliferation[29]; suppress interleukin 2 secretion[30]; and switch Th1 to Th2 type lymphocytes.[31] These findings suggest that NO acts as a self regulatory molecule limiting the Th1 immune response, thus downregulating local inflammation.[32] Whether iNOS plays a similar suppressive role in the course of Th1 associated liver injuries such as viral hepatitis or PBC remains questionable.

Taken together, our results show that the amounts of IFN-γ and iNOS transcripts are elevated in chronic HCV infection. Considering the high expression of IFN-γ and iNOS in HBV infection and also in PBC, the expression of these immunomodulatory molecules does not seem to be specific for HCV infection and HCV related liver damage. However, they may contribute to the pathobiology of Th1 mediated liver injuries such as HCV infection.

This work was supported by the Stiftung der Universität Göttingen. The authors thank Professor Dr L Füzesi, Department of Pathology, Division of Gastroenteropathology, University of Göttingen, and Mr A Fayazi, Washington University, for critical reading of the manuscript, and Mrs Adriana Soto for expert technical assistance.

1 Alter MJ, Margolis HS, Krawczynski K, *et al*. The natural history of community-acquired hepatitis C in the United States. *N Engl J Med* 1992;**327**:1899–905.
2 Di Bisceglie AM, Goodman ZD, Ishak KG, *et al*. Long-term clinical and histological follow-up of chronic posttransfusion hepatitis. *Hepatology* 1991;**14**:969–74.
3 Houghton M, Weiner A, Han J, *et al*. Molecular biology of the hepatitis C virus: implications for diagnosis, development and control of viral disease. *Hepatology* 1991;**14**:381–8.
4 Wejstal R. Immune-mediated liver damage in chronic hepatitis C. *Scand J Gastroenterol* 1995;**30**:609–13.
5 Mihm S, Hutschenreiter A, Fayyazi A, *et al*. High inflammatory activity is associated with an increased amount of IFN-γ transcripts in peripheral blood cells of patients with chronic hepatitis C virus infection. *Med Microbiol Immunol* 1996;**185**:95–102.
6 Napoli J, Bishop GA, McGuiness PH, *et al*. Progressive liver injury in chronic hepatitis C infection correlates with increased intrahepatic expression of Th1-associated cytokines. *Hepatology* 1996;**24**:759–65.
7 Bertoletti A, D'Elios MM, Boni C, *et al*. Different cytokine profiles of intrahepatic T cells in chronic hepatitis B and hepatitis C virus infections. *Gastroenterology* 1997;**112**: 193–9.
8 Toyonaga T, Hino O, Sugai S, *et al*. Chronic active hepatitis in transgenic mice expressing interferon-γ in the liver. *Proc Natl Acad Sci USA* 1994;**91**:614–18.
9 Ando K, Moriyama T, Guidotti LG, *et al*. Mechanisms of class I restricted immunopathology. A transgenic mouse model of fulminant hepatitis. *J Exp Med* 1993;**178**:1541–54.
10 Lyons CR. The role of nitric oxide in inflammation. *Adv Immunol* 1995;**60**:323–71.
11 Green SJ. Nitric oxide in mucosal immunity. *Nat Med* 1995; **6**:515–17.
12 Wood ER, Berger H Jr, Sherman PA, *et al*. Hepatocytes and macrophages express an identical cytokine inducible nitric oxide synthase gene. *Biochem Biophys Res Commun* 1993;**191**:767–74.
13 Mihm S, Fayyazi A, Ramadori G. Hepatic expression of inducible nitric oxide synthase transcripts in chronic hepatitis C virus infection: relation to hepatic viral load and liver injury. *Hepatology* 1997;**26**:451–8.
14 Desmet VJ, Gerber M, Hoofnagle JH, *et al*. Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatology* 1994;**19**:1513–20.
15 Knodell RG, Ishak KG, Black WC, *et al*. Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. *Hepatology* 1981;**1**:431–5.
16 Mihm S, Fayyazi A, Hartmann H, *et al*. Analysis of histopathological manifestations of chronic hepatitis C virus infection with respect to virus genotype. *Hepatology* 1997;**25**:735–9.
17 Breitschopf H, Suchanek G, Gould RM, *et al*. In situ hybridization with digoxigenin-labeled probes: sensitive and reliable detection method applied to myelinating rat brain. *Acta Neuropathol (Berl)* 1992;**84**:581–7.
18 Harada K, Van de Water J, Leung PSC, *et al*. In situ nucleic acid hybridization of cytokines in primary biliary cirrhosis: predominance of the Th1 subset. *Hepatology* 1997;**25**:791–6.
19 Bruch-Gerharz D, Fehsel K, Suschek C, *et al*. A proinflammatory activity of interleukin 8 in human skin: expression of the inducible nitric oxide synthase in psoriatic lesions and cultured keratinocytes. *J Exp Med* 1996;**184**:2007–12.
20 Radzun HJ, Hansmann ML, Heidebrecht HJ, *et al*. Detection of a monocyte/macrophage differentiation antigen in routinely processed paraffin-embedded tissues by monoclonal antibody Ki-M1P. *Lab Invest* 1991;**65**:306–15.
21 Dumoulin FL, Bach A, Leifeld L, *et al*. Semiquantitative analysis of intrahepatic cytokine mRNAs in chronic hepatitis C. *J Infect Dis* 1997;**175**:681–5.
22 Mosman TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol Today* 1996;**17**:138–46.
23 Zinkernagel RM. Protection and damage by antiviral immunity. *Harvey Lect* 1995;**89**:29–51.
24 Ando K, Guidotti LG, Wirth S, *et al*. Class I-restricted cytotoxic T lymphocytes are directly cytopathic for their target cells in vivo. *J Immunol* 1994;**152**:3245–53.
25 Denis M. Human monocytes/macrophages: NO or no NO. *J Leuk Biol* 1994;**55**:682–4.
26 Mannick JB, Asano K, Izumi K, *et al*. Nitric oxide produced by human B lymphocytes inhibits apoptosis and Epstein-Barr virus reactivation. *Cell* 1994;**79**:1137–46.
27 Majano PL, Garcia-Monzon C, Lopez-Cabrera M, *et al*. Inducible nitric oxide synthase expression in chronic viral hepatitis. *J Clin Invest* 1998;**101**:1343–52.
28 Taylor V, Miescher GC, Pfarr S, *et al*. Expression and developmental regulation of Ehk-1, a neuronal Elk-like receptor tyrosine kinase in brain. *Neuroscience* 1994;**63**:163–78.
29 Ianaro A, O'Donnell CA, di Rosa M, *et al*. A nitric oxide synthase inhibitor reduces inflammation, down-regulates inflammatory cytokines and enhances interleukin-10 production in carrageenin-induced oedema in mice. *Immunology* 1994;**82**:370–5.
30 Taylor-Robinson AW. Inhibition of IL-2 production by nitric oxide: a novel self-regulatory mechanism for Th1 cell proliferation. *Immunol Cell Biol* 1997;**75**:167–75.
31 Benbernou N, Esnault S, Shin HC, *et al*. Differential regulation of IFN-γ, IL-10 and inducible nitric synthase in T cells by cyclic AMP-dependent signal transduction pathway. *Immunology* 1997;**91**:361–8.
32 Taylor-Robinson AW. Counter-regulation of T helper 1 cell proliferation by nitric oxide and interleukin-2. *Biochem Biophys Res Commun* 1997;**233**:14–19.