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Inflammatory markers in chronic hepatitis C

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Abstract To test the hypothesis that inflammation in hepatitis C follows mechanisms common to immune-activated pathways, the distributions of T and B cells, adhesion molecules and transforming growth factor-β (TGF-β) were assessed in liver biopsies with chronic inflammation due to hepatitis C (HCV, *n*=8) and other causes (non-HCV, *n*=10). Frozen sections were immunostained using primary antibodies to CD2, CD20, CD4, CD8, intercellular adhesion molecule (ICAM-1), vascular cell adhesion molecule (VCAM)-1, HLA-DR, lymphocyte function-associated antigen (LFA)-1, and TGF-β. Inflammatory cells positive for each immunophenotypic marker were counted, and positive staining for adhesion molecules, HLA-DR and TGF β was graded in triads and lobules and compared in HCV and non-HCV biopsies. In all biopsies, T cells were more frequent than B cells, both in triads and lobules. CD20+, CD4+, CD8+ and LFA-1+ cells were increased in HCV compared to non-HCV biopsies. Portal lymphoid aggregates were present in 6 of 8 HCV biopsies and 3 of 10 non-HCV biopsies. Aggregates consisted of CD20+, CD4+, CD8+ and LFA-1+ cells, and ICAM-1 and VCAM-1 were increased. Sinusoidal lining cells in HCV biopsies and non-HCV biopsies with inflammation expressed HLA-DR, ICAM-1, and CD4. TGF-β was increased in foci of necrosis. Inflammation in chronic HCV involves common immune-mediated cellular effector pathways and the inflammation in the portal triads represents aggregation of both T and B cells, mediated in part by upregulation of adhesion molecules on portal stromal cells; this is possibly in response to antigens draining from necroinflammatory foci in the lobules. TGF-β is increased in active necroinflammatory foci, but not in portal lymphoid aggregates.

Key words Inflammatory cells · Adhesion molecules · Chronic hepatitis C

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Introduction

Previous immunopathological studies of liver biopsies from patients with chronic hepatitis have suggested that the distribution of inflammatory cells in chronic hepatitis C infection (HCV) is mediated by immune mechanisms common to many inflammatory processes. Increased T cytotoxic/suppressor cells are found in foci of necrosis in the lobules, and mixed populations of T helper/inducer cells and natural killer cells in the triads [5, 8, 11, 25]. Other studies showed that the portal lymphoid aggregates characteristic of HCV are composed of polymorphic populations of cells [10, 14] associated with upregulation of adhesion molecules [12, 19, 23] and neovascularization of triads [12]. It has been suggested that the lymphoid aggregates are similar to the primary follicles which form in lymph nodes after antigen stimulation [10]. The focus of previous studies has been to characterize the components of the inflammatory response in chronic hepatitis β (HBV) and HCV; comparisons with chronic hepatitis due to other causes have not been made.

The aim of this study was to use immunohistochemical techniques to study further the distribution of inflammatory cells, adhesion molecules and one inflammatory cytokine, transforming growth factor (TGF)-β, in HCV compared to hepatitis due to other causes. Our hypothesis was that the pattern of inflammation in HCV is not unique, and that we would find similarities in the distribution of inflammatory cells and mediators in the two groups. A second hypothesis was that the portal lym-

phoid aggregates represent regions of T and B cell accumulation mediated in part by increased expression of adhesion molecules and possibly cytokines such as TGF-β by portal stromal cells in response to antigens draining from necro-inflammatory foci in the lobules. That lymphoid aggregates are more frequent in HCV relates to the more extensive lobular damage in HCV compared with other disorders.

Materials and methods

Eighteen patients who had a liver needle biopsy performed at the University of Massachusetts Medical Center and had a portion of the biopsy frozen were studied. These patients included 8 with HCV, 10 who were negative for HCV and were biopsied for other reasons and 4 who were seronegative for HCV and had no inflammation in their biopsy. The 8 patients with HCV were all men, ranging in age from 33 to 48 years. All were seropositive for HCV by second generation enzyme immunoassay (EIA) and recombinant immunoblot assay (RIBA) tests and were referred to the University of Massachusetts Medical Center for evaluation for interferon-alpha (IFNα) therapy. Six patients had serum HCV RNA determination by the branched-chain DNA method (Quantiplex, Chiron). Of these, four patients had detectable viral levels in the serum, ranging from $4.1–272.4\times10^5$ Eq/ml (normal <3.5). Markers for hepatitis B were studied in seven patients; all were negative for HBsAg, three were positive and three were negative for anti-HBs. Three patients were negative for antibodies to hepatitis A. The patients had no other known causes of their liver disease; caeruloplasmin and alpha-1-antitrypsin levels were normal and titres for anti-smooth muscle antibodies were low or non-detectable. Haemochromatosis was excluded by examination of Prussian-blue stained sections of liver biopsies. Risk factors for HCV were intravenous drug use in three cases, and unknown in five cases. Seven patients had no known prior therapy for hepatitis. One patient had received standard dose IFN and IFN plus levamisole [4] 2 years prior to his biopsy, which was for relapse. The 2-year interval was considered long enough that the IFN would not affect the biopsy findings.

The ten patients without HCV included seven men and three women, ranging in age from 39 to 62 years. Seven patients were tested and were negative for serum antibodies to HCV (EIA II and/or RIBA II) and HBV; the other three patients were not tested because there was no clinical suspicion of HCV. The biopsy diagnoses were: alcoholism (three cases); primary sclerosing cholangitis (one case); primary biliary cirrhosis (two cases); haemochromatosis (one case); steatosis (one case); cirrhosis due to HBV (one case) and focal nodular hyperplasia (one case). In the last case, tissue adjacent to the focal nodular hyperplasia was studied. In six patients serum markers for other hepatotropic viruses were sought, and were negative. Biopsies from four patients seronegative for HCV which had no inflammation were included to provide a baseline for the distribution of markers in non-inflamed liver. The diagnoses in these cases were: congenital hepatic fibrosis, end stage; methotrexate toxicity, mild; normal liver, and steatosis.

All liver biopsies were performed using a standard Klatskin-modified Menghini needle. Samples 2–5 mm in length were snap frozen in liquid nitrogen and stored at −70° C. The biopsy samples for histologic evaluation and diagnosis were fixed in Carnoy's solution, embedded in paraffin, cut at 4 µm, and stained with haematoxylin and eosin, trichrome and Gomori's iron stain. For immunostaining, sections were cut at 4 µm, air-dried overnight at room temperature, then fixed in acetone for 10 min at room temperature and air-dried again. For staining, the slides were washed in phosphate-buffered saline (pH 7.4) containing 1% normal goat serum. Endogenous avidin and biotin were blocked using Vector's blocking kit. Endogenous peroxidase reactions were blocked using 3% hydrogen peroxide (H_2O_2) for 10 min. Sections were then incubated with the primary antibodies shown in Table 1 for 30 min, followed by a 30 min incubation with biotinylated horse anti-mouse (or rabbit) IgG (Vector Laboratories, Burlingame, Calif., USA), followed by a 30 min incubation with avidin horseradish peroxidase (Vector Laboratories). Reactive sites were revealed by incubation with 0.1 mg/ml 3,3´-diaminobenzidine-0.2% H_2O_2 followed by 2% copper sulphate treatment to enhance the colour of the reaction product. Sections were counterstained with haematox-

Table 1 Primary antibodies with their specificities, dilution and source (*NK* natural killer, *LFA* lymphocyte function-associated antigen, *ICAM* intercellular adhesion molecule, *VCAM* vascular cell adhesion molecule, *TGF-β* transforming growth factor β)

Antibody	Specificity	Dilution	Source
Immunophenotypic markers			
CD2	All T cells, NK cells	1:10	Becton-Dickinson
CD20	All B cells	1:400	Dako
CD4	Thelper/inducer cells	Neat	Becton-Dickinson
CD8	T cytotoxic/suppressor cells	1:1.5	Becton-Dickinson
Adhesion molecules			
CD11a (LFA-1)	Peripheral blood mononuclear cells	1:2,000	Becton-Dickinson
CD54 (ICAM-1)	Receptor for LFA-1 on endothelium, monocytes, fibroblasts, lymphocytes, B cells, myelocytes	1:2,000	Becton-Dickinson
Anti-VCAM-1	Receptor for lymphocytes and monocytes on endothelium, postcapillary venules, dendritic cells	1:2,000	Becton-Dickonson
HLA-DR	Class II antigen	1:25	Becton-Dickinson
Cytokine			
TGF-β	Peptide produced by platelets, activated monocytes/macrophages, hepatic stellate cells, Kupffer cells, endothelial cells	1:40	Oncogene Sci Incorporated

ylin. Positive controls were sections of fresh-frozen reactive lymph nodes. Negative controls were the liver biopsies run concurrently without the primary antibody.

Since the degree of inflammation and fibrosis were to be assessed in both HCV and non-HCV biopsies, we modified schemes previously proposed for chronic autoimmune and viral hepatitis [7, 17, 21] which could be applied to both groups. As in Knodell's system [17], piecemeal necrosis, portal inflammation and lobular inflammation were each graded separately, but with the following scale: 0 no inflammation; 1+ one focus of inflammation; 2+ a few foci of inflammation; 3+ many foci, diffuse in the biopsy. Fibrosis was assessed qualitatively as periportal fibrosis, bridging fibrosis or cirrhosis. Lymphoid aggregates, acidophilic bodies, cell swelling and steatosis were assessed as present or absent. All biopsies were evaluted without knowledge of the patient's diagnosis or HCV status.

For each antibody, one slide was scanned at 40× magnification and cells positive for each immunophenotypic marker were counted in lobules and in portal triads. The number of triads per biopsy section ranged from 3 to 10. To ensure that the fields counted were as equal as possible in their representation of triads or lobules, only high power fields (HPFs) in which a triad or lobule filled more than half of the field were counted. In triads with piecemeal necrosis, the edges of the triad were delineated arbitrarily by the hepatocytes in the limiting plate. Although periportal areas have been enumerated separately in some previous immunopathological studies [25], we found that the boundary between triad and lobule was frequently blurred in areas of piecemeal necrosis. Since hepatocytes must be recognized to identify piecemeal necrosis, we counted areas of piecemeal necrosis as lobular inflammation, and the cells on the triad side of these foci as portal. With these constraints, the number of positive cells per HPF in the triads and in the lobules was determined for each biopsy.

Since positive staining for adhesion molecules and TGF-β was more widespread, staining for these markers was evaluated qualitatively at 10× magnification and the distribution on stroma, bile ducts, sinusoidal lining cells and hepatocytes was noted. In both triads and lobules, positive staining was graded as 1+ if patchy, 2+ if present on less than half of the cells, and 3+ if present diffusely.

Biopsies from chronic HCV patients were compared with biopsies from non-HCV patients with respect to the frequency of positive staining for each marker in portal triads, and in the lobules. Differences in marker levels were evaluated using Kroskal Wallis one way analysis of variance by ranks. In the presence of a significant main effect, comparisons were made using pairwise Mann-Whitney U-tests with a Bonferroni adjustment to compensate for the additive type I error due to multiple comparisons.

Results

The grade of inflammation and degree of fibrosis for all biopsies are shown in Table 2. The biopsies with inflammation were comparable in the degree of inflammation, which was relatively mild. All had portal and lobular inflammation; piecemeal necrosis was found only in the biopsies from patients with viral hepatitis and PBC. Portal lymphoid aggregates were present in 6/8 HCV biopsies, 3/10 non-HCV biopsies with inflammation, and none of the non-inflamed, non-HCV biopsies. In the samples frozen for immunostaining, lymphoid aggregates were present in 4/8 HCV and 3/10 non-HCV biopsies.

Compared with the non-HCV, non-inflamed biopsies, all of the biopsies with inflammation showed an increase in cells positive for CD2, CD20, CD4, CD8 and lymphocyte function-associated antigen (LFA)-1. There were significant differences ($P<0.05$) between controls and the biopsies with inflammation for CD2 in both triads and

Table 2 Diagnosis, grade of inflammation and degree of fibrosis in chronic hepatitis C (HCV) and non-HCV biopsies (*M* male, *F* female, *Chr.* chronic, *HBV* hepatitis B virus)

Patient details (age, sex)	Diagnosis	Inflammatory score[a]	Lymphoid	
			aggregates	fibrosis
HCV, inflammation (*n*=8)				
41 M	Chr. hepatitis	2	Yes	Periportal
44 M	Chr. hepatitis	5	Yes	Periportal
38 M	Chr. hepatitis	5	Yes	Periportal
35 M	Chr. hepatitis	3	No	Periportal
33 M	Chr. hepatitis	2	No	Bridging
48 M	Chr. hepatitis	4	Yes	Periportal
33 M	Chr. hepatitis	3	Yes	0
33 M	Chr. hepatitis	7	Yes	Bridging
non-HCV, inflammation (*n*=10)				
57 M	Cirrhosis due to HBV	6	No	Cirrhosis
62 M	Haemochromatosis	2	No	0
46 M	Alcoholic liver disease	5	Yes	Cirrhosis
53 F	Primary biliary cirrhosis	4	No	0
47 F	Alcoholic liver disease	3	No	0
39 M	Focal nodular hyperplasia	1	Yes	0
54 M	Alcoholic liver disease	1	No	0
40 F	Primary biliary cirrhosis	4	Yes	0
61 M	Primary sclerosing cholangitis	2	No	0
55 M	Steatosis	2	No	0
non-HCV, no inflammation (*n*=4)				
5 M	Congenital hepatic fibrosis	0	No	Cirrhosis
37 F	Haemosiderosis	0	No	0
74 F	Congestion	0	No	0
45 M	Steatosis	0	No	0

[a] Based on grading schemes of Knodell [17] and Desmet et al. [7]. The inflammatory score represents the sum of the grades for piecemeal necrosis, lobular inflammation and portal inflammation

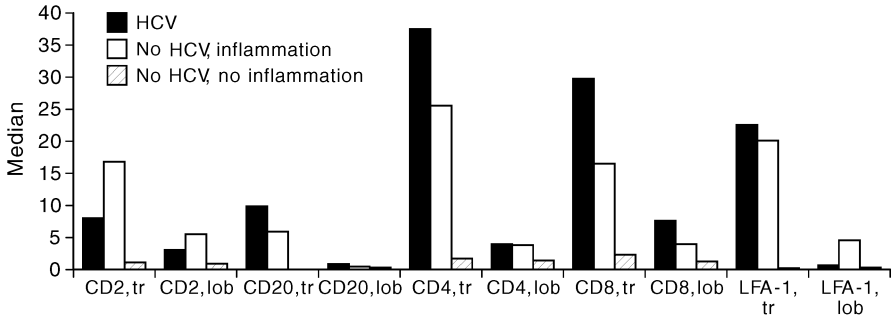

Fig. 1 Graph showing the median numbers of cells per high power field (HPF) staining positive for CD2, CD20, CD4, CD8 and lymphocyte function-associated antigen (LFA)-1 in triads (*tr*) and lobules (*lob*) in biopsies from chronic hepatitis C (HCV)-positive patients (*n*=8), and HCV-negative patients with (*n*=10) and without (*n*=4) inflammation

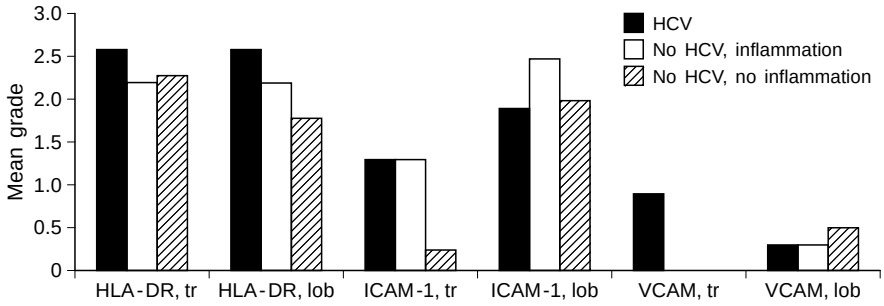

Fig. 2 Graph showing the median numbers of cells per HPF staining positive for HLA-DR, intercellular adhesion molecule (ICAM)-1, and vascular cell adhesion molecule (VCAM)-1 in triads (*tr*) and lobules (*lob*) in biopsies from HCV-positive patients (*n*=8), and HCV-negative patients with (*n*=10) and without (*n*=4) inflammation

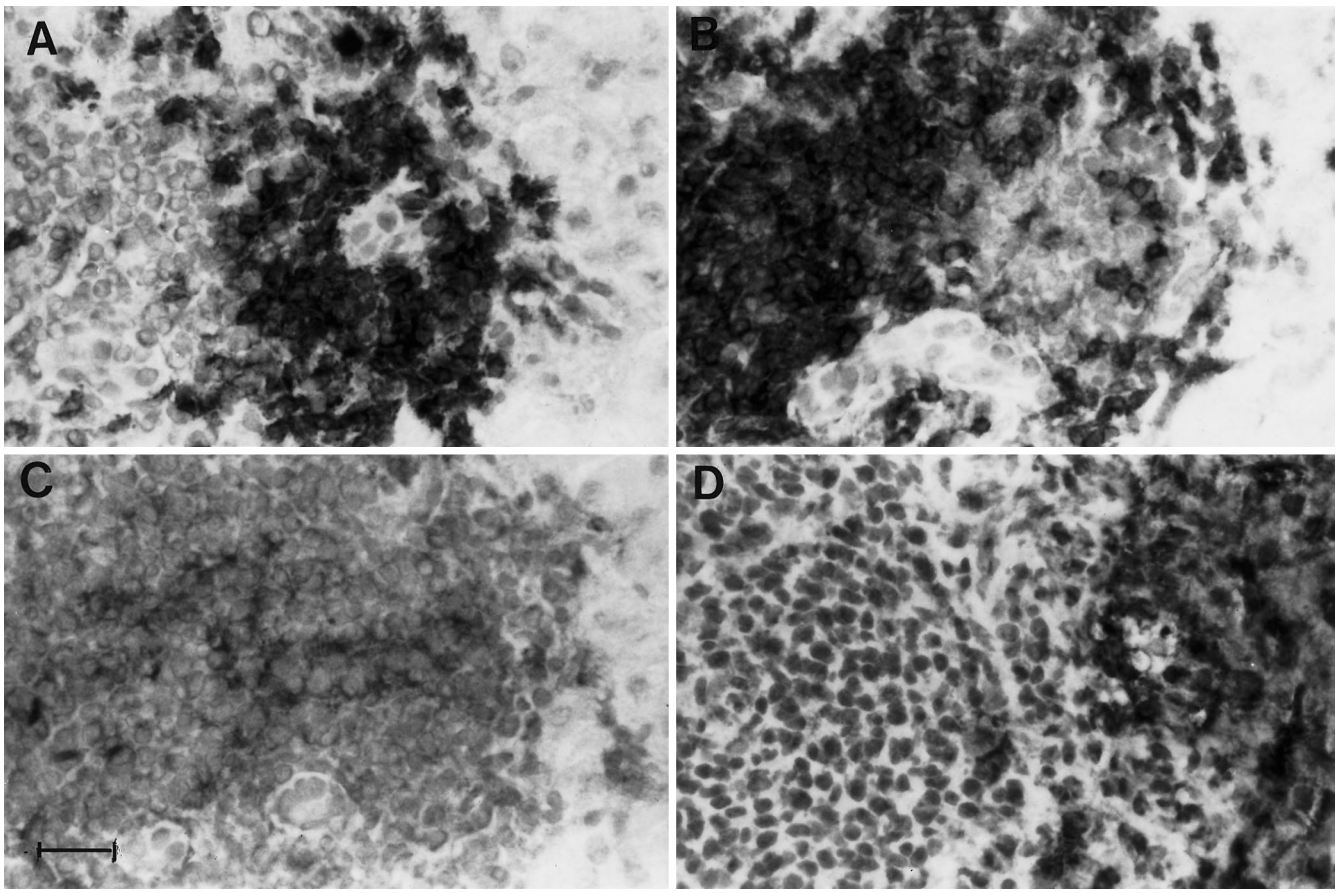

Fig. 3A–D Photomicrographs showing the same triad in a biopsy from a patient with HCV immunostained for CD20 (**A**), CD4 (**B**), ICAM-1 (**C**) and transforming growth factor (TGF)-β1 (**D**). The lymphoid aggregate is composed of CD20+ and CD4+ cells. ICAM-1 is present. TGF-β1 is expressed in piecemeal necrosis at the edge of the triad. (×400, *bar*=100 μm)

lobules, CD20 in triads, CD4 in triads and lobules and CD8 in triads. CD20+, CD4+, CD8+, and LFA-1+ cells were increased in the HCV biopsies compared with the non-HCV biopsies, but there were no significant differences in the group means between these two groups. Me-

dians of the frequencies of the cells positive for the immunophenotypical markers are shown in Fig. 1. In both HCV and non-HCV biopsies, the lymphoid aggregates, when present, were composed of CD20+, CD4+ and LFA-1+ cells, surrounded by CD8+ cells.

HLA-DR and intercellular adhesion molecule (ICAM)-1 were present in non-inflamed, non-HCV biopsies, particularly in the lobules. As shown in Fig. 2, HLA-DR was strongly expressed in both triads and lobules in all biopsies. ICAM-1 was increased in the triads in inflamed biopsies compared to the non-inflamed biopsies. Vascular cell adhesion molecule (VCAM)-1 was present in triads only in the HCV biopsies, and it was associated with the lymphoid aggregates.

Positive staining for TGF-β was detected in all biopsies and was similar in HCV and non-HCV biopsies. In the triads, positive staining for TGF-β was either around the edges, or diffuse throughout the triads. In the lobules, sinusoidal lining cells were positive. There was no particular distribution in the three zones. There was no correlation between the amount of positive staining for TGF-β and the grade of inflammation or fibrosis. However, qualitatively TGF-β was increased in areas of piecemeal necrosis, and lymphoid aggregates were negative. Examples of positive immunostaining for CD20, CD4, ICAM-1 and TGF-β in a single triad in an HCV patient are shown in Fig. 3A–D.

Discussion

This study compared markers of the inflammatory response in chronic hepatitis C and chronic hepatitis due to other causes. Inflammatory markers which could be detected by immunohistochemical techniques were studied, including mrkers for T and B cells (CD2, CD20, CD4, CD8), lymphocyte activation marker LFA-1, adhesion molecules ICAM-1 and VCAM-1, MHC Class II antigen HLA-DR, and a cytokine, TGF-β, which may recruit inflammatory cells into areas of acute injury [12, 15]. The results showed no significant differences between the biopsies with and without HCV in the distribution of T and B cells in the triads and lobules. In all of the biopsies both T cells and B cells were more frequent in the triads than in the lobules. However, the HCV biopsies showed the highest frequencies of CD20+, CD4+, CD8+, and LFA-1+ cells in the triads, because they were concentrated in the lymphoid aggregates, which were more frequent in the HCV group. There was also a marked increase in expression of ICAM-1 in the triads in all inflammed biopsies; increased VCAM-1 was found only in the HCV biopsies. These finding support the suggestions from previous studies that the inflammation in the triads represents organized proliferation centres of T and B cells associated with upregulation of adhesion molecules on cells in the triads in response to antigens from inflammation in the lobules. TGF-β is increased in active necroinflammatory foci, but not in the portal lymphoid aggregates.

Previous immunopathological studies of patients with HCV described predominantly T cytotoxic/suppressor (CD8+) cells in the lobules and a mixed population of T helper/inducer (CD4+) and NK cells in the triads [5, 8, 11, 13, 25]. Ultrastructural studies have shown activated T cells in direct contact with hepatocytes [8]. The prevailing theory is that the virus and/or cell debris containing viral antigens are phagocytosed by Kupffer cells and then HCV-derived antigenic peptides are presented with HLA Class II antigens on the cell surface [13]. This activates CD4+ cells which initiate both T and B cell effector responses. HCV may be one of many potential triggers for the T and B inflammatory cell pathways. Thus the inflammatory reaction seen in liver biopsies ought to be similar in chronic hepatitis due to HCV and other causes. In fact, numerous histological studies have shown features common to HCV, HBV and autoimmune hepatitis [1, 6, 16, 21, 24], but comparisons between viral and non-viral hepatitis other than autoimmune hepatitis have not been made.

The two groups compared in this study were alike in so far as their hepatitis was chronic, as documented clinically, and the biopsies revealed chronic inflammation. Inflammation was graded using a modification of previous schemes [7, 17] which could be applied in all of our biopsies. Inflammation was mild to moderate in both HCV and non-HCV groups. The mean score in the HCV group was 4, and in the non-HCV group with inflammation it was 3. None of the biopsies had a maximum score of 9. The highest score, 7, was in the HCV group. Lymphoid aggregates were found in both groups but they were more common in the HCV group.

Lymphoid aggregates are characteristic of HCV infection [6, 16, 21]. One underlying hypothesis of our study was that the portal lymphoid aggregates represent proliferation centres of both T and B cells forming in response to antigens draining into the triads from necroinflammatory foci in the lobules. This implies that portal triad cells capable of antigen presentation are involved in the formation of the aggregates. Previous studies have compared the lymphoid aggregates to the primary and secondary follicles which develop in lymph nodes reacting to antigen stimulation [10]. Fully developed lymphoid aggregates have been found to exhibit a germinal centre of active B cells and a dendritic reticulum cell network surrounded by T lymphocytes organized in zones of helper, cytotoxic/suppressor and activated lymphocytes [19]. It follows that as in lymph nodes, upregulation of adhesion molecules for trapping and recruiting inflammatory cells might regulate the formation of the lymphoid aggregates in the liver in hepatitis.

In the current study, as in previous studies, the portal lymphoid aggregates were composed of CD20+ and CD4+ cells, surrounded by CD8+ cells. This organization was difficult to assess, but in some biopsies the plane of section appeared to cross the centre of an aggregate, and an arrangement with CD20+, CD4+ and LFA-1+ cells in the centre surrounded by CD8+ cells was present. ICAM-1 was expressed in the centre of the ag-

gregates. ICAM-1 is a glycoprotein expressed on a wide variety of cells, including endothelial cells, keratinocytes, fibroblasts, lymphocytes and myelocytes [3, 22]. Its expression can be upregulated by mediators such as IFNγ, tumour necrosis factor, and interleukin-1, suggesting that it plays a role in inflammation [9]. VCAM-1 is a glycoprotein which is expressed on the surface of stimulated endothelial cells. It is an adhesion molecule for many different cell types, including lymphocytes, monocytes, neural cells and primitive haematopoietic cells. LFA-1 is a member of the beta 2 integrin family. It is the ligand for ICAM-1 and is expressed widely on the surface of peripheral blood leukocytes to promote the binding of lymphocytes in lymph nodes and mucosal lymphoid tissues for lymphocyte homing and T-cell migration to inflamed tissues [3, 22]. In the normal liver, ICAM-1 is expressed only on sinusoidal lining cells and VCAM-1 is not expressed [23]. Both adhesion molecules are upregulated in both triads and sinusoids in acute and chronic inflammation of the liver [23]. In one study of patients with HCV, ICAM-1, HLA-DR and LFA-3 were expressed on hepatocytes in regions with piecemeal necrosis and intralobular clusters. Induction of VCAM-1 and other adhesion molecules in portal triad vessels [12, 23], and neovascularization in the triads [12] have also been described in HCV. Of interest in our cases was the increase in LFA-1+ cells in the triads in HCV biopsies compared to non-HCV biopsies, as LFA-1 is the receptor for ICAM-1 [22]. The demonstration of increased LFA-1+ cells, and ICAM-1 and VCAM-1 expression in triads in inflammation in this and previous studies [12, 19, 23] supports the notion that these adhesion molecules can be up-regulated in response to antigenic stimulation, and they are involved in the recruitment of inflammatory cells into the triads. That they are more frequent in HCV than in other forms of hepatitis may relate to the fact that hepatocyte damage is ongoing with continuous antigenic stimulation of the portal triad cells.

We expected that TGF-β would be increased in all biopsies with inflammation, and in the lymphoid aggregates. Low levels of TGF-β1 transcripts have been found in normal portal stromal cells using in-situ hybridization techniques [18]. After acute injury in the liver; TGF-β is produced by the inflammatory cells, later by stellate cells, endothelial cells and, possibly hepatocytes, as repair processes develop [15]. Since production of TGF-β by the hepatic non-parenchymal cells is markedly increased after liver injury at both the protein and mRNA levels [2, 18, 20], it is felt that TGF-β may be involved in recruitment of inflammatory cells into areas of injury. It is also a promotor of extracellular matrix production, and is thought to play a major role in liver regeneration and fibrogenesis [2]. We found that TGF-β was not associated with the lymphoid aggregates or the areas of upregulation of adhesion molecules. We did find a strong association of TGF-β with areas of active lobular and piecemeal necrosis, in accord with the findings of Inuzuka et al. [15]. We had too few biopsies with fibrosis to compare fibrotic and non-fibrotic livers for distribution of TGF-β.

In summary, we used immunohistochemical techniques to study the distribution of inflammatory cells in liver biopsies from patients with HCV and found increased CD20+ and CD4+ cells associated with upregulation of ICAM-1 and VCAM-1 in the portal lymphoid aggregates. This profile, although not specific for HCV, was more frequent in the biopsies from patients with HCV than in biopsies of inflammatory diseases of other aetiologies. The findings support the hypothesis that the inflammation in the portal triads represents proliferation centres of T and B cells which are highly-organized, antigen-driven, and mediated in part by upregulation of adhesion molecules on portal stromal cells. TGF-β is increased in active necroinflammatory foci, but not in the portal lymphoid aggregates.

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