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$CD4^+/CD8^+$ ratio of liver-derived lymphocytes is related to viraemia and not to hepatitis C virus genotypes in chronic hepatitis C

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SUMMARY

The pathogenic mechanisms that lead to chronic hepatitis C are unknown. As hepatitis C virus (HCV) has been shown to induce T cell response, we assessed whether a particular T lymphocyte subset could be preferentially detected in the liver of patients with chronic hepatitis C in relation to viraemia or HCV genotypes. The immunophenotypes of liver-derived lymphocytes were analysed in 26 patients by flow cytometry and immunohistochemistry. Viraemia was quantified by branched DNA assay. Using this assay, HCV RNA was not detectable in six patients. HCV RNA was detected in 20 patients, and titres ranged from 8 to 137×10^6 Eq/ml. Genotyping was performed using a line probe assay. Type 1a, 1b, 2a, 3a and 4a were found to infect 2, 10, 2, 7 and 3 patients, respectively. The $CD4^+/CD8^+$ ratio of liver-derived lymphocytes was significantly higher ($P < 0{\cdot}01$) in patients with detectable viraemia than in patients without detectable viraemia. In contrast, neither the percentage of γ/δ T lymphocytes nor that of $CD2^+CD57^+$ cells was different in the groups. When comparing the $CD4^+/CD8^+$ ratio, the percentage of γ/δ T lymphocytes or $CD2^+CD57^+$ cells according to genotype, the differences were not significant. These results suggest that the $CD4^+/CD8^+$ ratio of liver-derived lymphocytes is related to viraemia but not to HCV genotypes in patients with chronic hepatitis C, and that T lymphocytes may be involved in the pathogenesis of liver lesions in chronic hepatitis C.

Keywords hepatitis C virus chronic hepatitis C liver-derived lymphocytes lymphocyte subsets viraemia hepatitis C virus genotype

INTRODUCTION

Hepatitis C Virus (HCV) is the major cause of parenteral non-A, non-B hepatitis [1,2]. Nucleotide sequence differences of both the coding and non-coding region detected in the virus isolates have led to a classification of HCV into several genotypes [3–8]. Whether particular genotypes of HCV are related to liver disease severity, infectivity and response to antiviral therapy remains to be clarified. HCV infection is usually diagnosed by assays which detect antibodies to HCV antigens. However, the presence of anti-HCV antibodies may not give information about viral replication. Serum HCV RNA detection provides direct evidence for active viraemia. Viraemia quantification may be assessed by a new assay that uses a branched DNA (bDNA) signal amplification [9]. It has been suggested that quantitative study of HCV RNA may be useful since patients with low HCV viraemia are likely to respond to interferon therapy [10].

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Development of a chronic liver disease occurs in at least 50% of infected patients [11]. Chronic hepatitis C is histologically defined by the association of mononuclear cell infiltrates as well as hepatocellular necrosis and fibrosis whose severity depends on liver disease stage and activity. The pathogenic mechanisms that result in chronic HCV hepatitis remain unknown. On one hand, the cytopathic nature of the HCV virus is questioned. On the other hand, the role of the immune response in tissue damage must also be considered. The balance between the host's immune response and the virus is complex. In some viral infections, cytotoxic T cell responses may elicit viral clearance or, on the contrary, may be involved in the immunopathogenesis of the disease [12,13]. In HCV infection, experimental infection of chimpanzees has shown that HCV-specific cytotoxic T cells derived from the liver lyse cells expressing viral proteins [14]. In patients with chronic hepatitis C, HCV-specific T lymphocytes have been found in the peripheral blood, and HCV cytotoxic T cell response has been found to be MHC class I-restricted [15,16]. In addition, several studies have shown that HCV-specific T lymphocytes

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are present among liver-derived lymphocytes [17,18], thereby emphasizing that T cell response may be a main counterpart of the immune response to HCV infection.

The aim of this study was to assess whether a specific T lymphocyte subset could be associated with either viraemia or the genotype of HCV in patients with chronic hepatitis C. Immunophenotypes of liver-derived lymphocytes and peripheral blood lymphocytes from patients with different levels of viraemia and genotypes were analysed by flow cytometry. Surface markers such as CD8, γ/δ T cell receptor or CD57 expressed on potential cytotoxic cells were studied. In addition, results obtained by flow cytometry were compared with those obtained using conventional immunohistochemical techniques. The CD4$^+$/CD8$^+$ ratio of liver-derived lymphocytes, but not the percentage of T cells bearing the γ/δ receptor or that of CD2$^+$CD57$^+$ cells, appears to be related to viraemia but not to HCV genotypes.

PATIENTS AND METHODS

This study included 26 patients (13 males and 13 females) ranging in age from 20 to 62 years. All patients had chronic hepatitis C diagnosed on the basis of their clinical data, aminotransferase levels, serological markers (see below) and histological data (Table 1). None of the patients had cirrhosis. Disease activity was graded according to a numerical scoring system developed by Knodell *et al.* [19].

All subjects had anti-HCV antibodies, and were negative for hepatitis B surface antigen and anti-HIV antibodies. Patients were investigated for serum HCV RNA quantification and HCV type infection (Table 2), then divided into two groups according to viraemia. Group I included six patients with no detectable serum HCV RNA as determined by bDNA assay at the time of their liver biopsy. Group 2 included 20 patients with positive serum HCV RNA at the time of their liver biopsy.

All patients underwent needle liver biopsy as part of their diagnosis evaluation. In all cases, one single pass was necessary to collect liver tissue. No patient was undergoing anti-viral therapy at the time of the liver biopsy.

This study was performed according to the ethical guidelines of the 1975 declaration of Helsinki, and patients gave their informed consent for liver biopsy.

Table 1. Histological features of the patients

Patient	Sex/age (years)	Serum alanine aminotransferase	Components of the histological activity index				Knodell score
			PMN†	IL‡	P§	F¶	
1	F, 54	4*	1	3	3	1	8
2	F, 26	5	1	3	1	0	5
3	F, 33	2	1	0	1	0	2
4	F, 28	⩽1	1	1	1	0	3
5	M, 26	2	1	1	1	0	3
6	M, 42	⩽1	1	1	3	0	5
7	F, 52	⩽1	3	3	3	0	9
8	F, 28	2	0	1	3	1	5
9	F, 38	⩽1	3	1	3	0	7
10	M, 26	4	1	0	1	3	5
11	F, 20	⩽1	1	1	1	0	3
12	M, 45	4	3	3	3	0	9
13	F, 31	3	1	1	1	1	4
14	F, 42	3	4	3	4	1	12
15	M, 41	⩽1	1	1	3	0	5
16	F, 39	2	4	1	4	3	12
17	M, 31	13	3	3	3	1	10
18	M, 39	2	3	1	3	1	8
19	M, 37	4	3	1	3	3	10
20	M, 62	4	3	3	4	3	13
21	F, 59	5	1	1	1	1	4
22	M, 35	4	3	1	3	3	10
23	M, 56	9	3	4	4	3	14
24	F, 56	2	3	1	3	3	10
25	M, 35	⩽1	1	3	1	1	6
26	M, 58	4	3	3	4	1	11

* Serum alanine aminotransferase levels are expressed as multiple of the normal value cutoff.

† Piece meal necrosis.

‡ Intralobular degeneration and focal necrosis.

§ Portal inflammation.

¶ Fibrosis.

Patients were listed according to their viraemia level (1 to 6, bDNA HCV RNA negative; 7 to 26, bDNA HCV RNA positive) as reported in Table 2. The activity of the liver disease was evaluated according to Knodell score [19].

Peripheral blood

Serum and heparinized blood samples were collected from all patients on the day of their liver biopsy. Samples were aliquoted and stored at −20°C until analysed, whereas heparinized blood samples were used within 24 h of collection.

HCV markers. Antibodies to HCV were detected using third-generation ELISA assay (Abbott Laboratories, Chicago, IL) and recombinant immunoblot RIBA-3 assay (Ortho Diagnostic System, Raritan, NJ).

HCV RNA detection was performed with reverse transcriptase 'nested' polymerase chain reaction (PCR) as previously described [20]. Briefly, RNA was extracted from 100 μl of serum stored at −20°C. We used two sets of synthetic oligonucleotide primers derived from the 5′ non-coding region of the HCV genome. These primers directed the amplification of 277 bp and 177 bp fragments, respectively.

Serum HCV RNA was measured using the bDNA signal amplification assay (Quantiplex; Chiron Corporation, Emeryville, CA) [21]. This assay is based on the specific hybridization of synthetic oligonucleotides to the 5′ untranslated (UTR) region and core genes of HCV RNA, which allow the RNA to be captured onto the surface of a well in a microwell plate. Synthetic bDNA molecules and multiple copies of an alkaline phosphatase-linked probe are hybridized to the immobilized complex (signal amplification). Detection is achieved by incubating the complex with a chemiluminescent substrate (Dioxetane) and measuring the light emission generated by the bound alkaline phosphatase. Each serum sample was run in duplicate (50 μl per assay). The limit of detection of the assay was $3{\cdot}5 \times 10^5$ copies HCV genome equivalent per ml (Eq/ml).

HCV genotyping was performed using an assay (Inno-LiPA; Innogenetics, Gent, Belgium) based on the reverse-hybridization principle [22]. Labelled PCR products were hybridized to specific oligonucleotides directed against the variable regions of 5′ UTR, immobilized as parallel lines on membrane strips. During nested PCR, the product was biotinylated, which allowed detection of hybrids by alkaline phosphatase-labelled streptavidin. According to Simmonds' classification [23], this assay allowed a determination of HCV types 1 to 5 and subtypes 1a, 1b, 2a, 2b, 3a and 3b starting from the 5′ UR amplified products.

Peripheral blood mononuclear cell analysis. Whole blood samples were prepared and analysed using the Coulter Q-Prep automated lysing system (Coulter Immunology, Hialeah, FL): whole blood and MoAbs were incubated at room temperature for 15 min and lysed according to Q-Prep protocol, then kept at 4°C until analysed by flow cytometry. The following MoAbs were used: FITC-conjugated CD3 (Biomedex, Nice, France); RPE-conjugated CD4 and FITC-conjugated CD8; the latter MoAbs were combined into a dual-colour reagent assay (Biomedex). MoAbs were used at the manufacturer's recommended concentrations.

Table 2. Virological status of the patient

Patient	Sex/age (years)	Source of infection	Duration of disease (years)	HCV RNA 10^5 Eq/ml	Genotype
1	F, 54	PT*	6	—	4a
2	F, 26	Others	1	—	3a
3	F, 33	IVDA†	2	—	4a
4	F, 28	Others	7	—	1a
5	M, 26	IVDA	2	—	3a
6	M, 42	Others	3	—	ND‡
7	F, 52	IVDA	10	8·63	1b
8	F, 28	Sporadic	2	9·57	1b
9	F, 38	IVDA	10	10·11	1b
10	M, 26	IVDA	2	17·10	3a
11	F, 20	Sporadic	2	17·54	1b
12	M, 45	IVDA	4	20·11	4a
13	F, 31	PT	2	23·93	1b
14	F, 42	Others	5	27·70	1b
15	M, 41	Sporadic	1	33·56	2a
16	F, 39	PT	2	39·70	3a
17	M, 31	IVDA	1	51·31	3a
18	M, 39	IVDA	4	63·66	2a
19	M, 37	IVDA	2	66·04	3a
20	M, 62	PT	32	71·16	1a
21	F, 59	PT	10	76·45	1a 1b 2a
22	M, 35	IVDA	2	78·95	3a
23	M, 56	Sporadic	3	96·12	1b
24	F, 56	PT	31	115·4	1b
25	M, 35	IVDA	5	117·2	1b
26	M, 58	Sporadic	2	137·1	1b

* Post-transfusion.

† Intravenous drug addict.

‡ Not determined since reverse transcription polymerase chain reaction was negative.

Liver specimens

Liver biopsy specimens were obtained with disposable Menghini biopsy needles, diameter 1·8 mm (B. Braun Melsungen AG, Melsungen, Germany). Each liver specimen was divided into three parts: one part was fixed in 10% buffered formaldehyde–saline and processed for routine histopathological examination. One part was snap-frozen in liquid nitrogen for immunohistochemical techniques. One part was collected in HEPES-buffered RPMI 1640 medium (Eurobio, Paris, France) containing 5% (v/v) heat-inactivated fetal calf serum (FCS; Eurobio) for flow cytometry analysis.

Flow cytometry. The medium was removed and tissue samples were incubated in 1 ml of new RPMI 1640 medium to avoid blood contamination. The size of biopsy specimens ranged from 5 to 10 mm. Samples were mechanically disrupted using pincers. Cell suspension was obtained by passing the tissue through a fine needle several times using a syringe followed by passage through a nylon mesh filter to remove the undissociated tissue. No additional lymphocyte isolation procedure was utilized to avoid bias from cell loss.

Double immunolabelling experiments were systematically performed; RPE-conjugated anti-CD2 (Coulter) was combined with the following FITC-conjugated antibodies: anti-CD3 (Biomedex), anti-CD4 (Biomedex), anti-CD8 (Biomedex), anti-α/β T cell receptor (TCR) (Becton Dickinson, Palo Alto, CA), anti-γ/δ TCR (Coulter), anti-CD57 (Immunotech, Marseille, France). Cell suspension (100 μl) was incubated with 10 μl of MoAbs for 30 min at 4°C. The cells were then washed in RPMI 1640 medium and fixed in 1% formaldehyde solution before analysis.

Two-colour analysis was performed in a FACStar Plus (Becton Dickinson). Gating was performed using the forward scatter *versus* 90° angle scatter (side scatter). One thousand to 3000 cells selected from this gate were analysed. To partially avoid the background fluorescence related to autofluorescent cells, a trigger was used on red fluorescence. Only cells that were positively discriminated by the presence of CD2 surface marker were further analysed for the presence of CD3, CD4, CD8, α/β TCR, γ/δ TCR or CD57, respectively. For all patients, the percentages of $CD2^+CD3^+$, $CD2^+CD4^+$, $CD2^+CD8^+$, $CD2^+$ γ/δ TCR^+, $CD2^+CD57^+$ liver-derived lymphocytes were estimated and recorded. The data were analysed using Lysis II software (Becton Dickinson).

Immunohistochemistry. Serial sections from frozen tissue blocks were cut and stained with the following MoAbs: anti-CD3, anti-CD4, anti-CD8 (Becton Dickinson) with a three-step immunoperoxidase technique, as described by Mason *et al.* [24]. Sections (4 μm thick) were dried overnight at room temperature and fixed for 10 min in acetone immediately before use. After rehydration in TBS, tissue sections were sequentially incubated for 40 min with peroxidase-labelled species-specific immunoglobulin antibodies. Peroxidase-labelled rabbit anti-mouse immunoglobulin (Dako, Copenhagen, Denmark) and swine anti-rabbit immunoglobulin (Dako) were diluted 1:20 in TBS. The colour reaction product was developed according to the method of Graham & Karnovski [25]. A light nuclear countercoloration was obtained by staining sections with Harris' haematoxylin for 60 s.

Statistical analysis

The non-parametric Wilcoxon test was used to compare the $CD4^+/CD8^+$ ratio obtained by flow cytometry with those obtained using immunohistochemical techniques.

The non-parametric Mann–Whitney *U*-test was used to compare the group 1 with the group 2, whereas the non-parametric Kruskall–Wallis test was used to compare the group A with the group B and the group C.

RESULTS

Histological features

The Knodell score was assessed for each patient. Scores ranged from 2 to 14 (Table 1). When the first three components of the histological activity index were taken into account [26], seven patients had minimal chronic hepatitis, 11 had mild chronic hepatitis and eight had moderate chronic hepatitis.

Virological status

Amplification of HCV RNA by means of PCR was successful in samples from 25 of 26 patients.

Viraemia quantification and HCV genotypes were investigated for all patients and reported in Table 2.

Viraemia quantification. In six patients (group 1), serum HCV RNA was not detectable using bDNA assay. However, HCV RNA could be detected in the serum of five out of six of these patients by PCR. In 20 patients (group 2), serum HCV RNA was detected at levels ranging from $8{\cdot}63 \times 10^5$ to $137{\cdot}1 \times 10^5$ Eq/ml (median 45·51).

The Knodell score ranged from 2 to 8 (median 4) and from 3 to 14 (median 9) in group 1 and group 2, respectively. It was significantly higher in group 2 than in group 1 (Mann–Whitney *U*-test: U1 = 101·5, $P < 0{\cdot}05$).

There was no correlation between serum HCV RNA levels and aminotransferase levels.

HCV genotypes. Type 1a, 1b, 2a, 3a and 4a were found to infect two, 10, two, seven and three patients, respectively. HCV genotype could not be determined in one patient (patient 6) because of a negative PCR. One patient (patient 21) appeared to be multi-infected by HCV type 1a, 1b and 2a. The patients were divided into three groups according to genotypes to allow statistical analysis despite distribution imbalance. Group A included the 10 patients infected by HCV type 1b. Group B included the seven patients infected by HCV type 3a, and group C included the seven patients infected by HCV type 1a, 2a or 4a. The multi-infected patient was not included in any group.

The Knodell score ranged from 3 to 14, from 3 to 12 and from 2 to 13 in group A, group B and group C, respectively. There was no significant difference between the three groups.

Regarding serum HCV RNA levels, there was no significant difference between the three groups.

Analysis of surface markers

Analysis of liver-derived lymphocytes. The liver-derived lymphocytes were studied by two-colour flow cytometry. Only $CD2^+$ cells were considered for the expression of CD3, CD4, CD8, α/β TCR, γ/δ TCR or CD57. Counts of $CD2^+$ $CD4^+$, $CD2^+CD8^+$, $CD2^+$ α/β TCR^+ and $CD2^+$ γ/δ TCR^+ cells were referred to the count of $CD2^+CD3^+$ cells and the $CD4^+/CD8^+$ ratio of liver-derived lymphocytes was calculated for all liver specimens.

According to viraemia, the $CD4^+/CD8^+$ ratios ranged from 0·48 to 0·99 (median 0·51) in group 1. In group 2, the $CD4^+/$

Table 3. T cell subpopulations of liver lymphocytes of the patients

Patient	Sex/age (years)	Flow cytometry analysis: Per cent of $CD2^+CD4^+$ cells	Per cent of $CD2^+CD8^+$ cells	$CD4^+/CD8^+$ ratio	Per cent of $\gamma\delta$ T cells	Per cent of $CD2^+CD57^+$ cells	Immunohistochemical analysis $CD4^+/CD8^+$ ratio
1	F, 54	33·8	69·8	0·48	8·6	2·8	
2	F, 26	33·2	61·6	0·53	3·4	2·7	597/772 = 0·77
3	F, 33	29·8	58·3	0·51	13·2	11·7	(298/218) = NC
4	F, 28	30·3	60·3	0·50	4·1	2·5	(397/311) = NC
5	M, 26	49·2	49·6	0·99	ND	ND	743/489 = 1·51
6	M, 42	22·9	48·5	0·47	9·2	4·7	346/207 = 1·67
7	F, 52	60·5	38·3	1·53	12·2	5·9	489/521 = 0·93
8	F, 28	23·9	54·4	0·43	9·9	ND	
9	F, 38	43·9	32·3	1·35	ND	ND	
10	M, 26	22·3	41·1	0·54	3·7	3·8	983/432 = 2·27
11	F, 20	35·2	46·7	0·75	4	3·6	668/718 = 0·93
12	M, 45	44·7	51·2	0·87	4·2	ND	
13	F, 31	69·1	41·1	1·68	ND	ND	258/393 = 0·65
14	F, 42	59·7	54·9	1·08	1·5	2·5	1 219/697 = 1·74
15	M, 41	51·3	30·4	1·68	3·5	3·3	649/207 = 3·13
16	F, 39	45·2	59·7	0·75	7·1	3·9	1 160/745 = 1·55
17	M, 31	59·5	40·1	1·47	ND	ND	572/1 220 = 0·46
18	M, 39	48·7	70·2	0·69	ND	ND	(188/373) = NC
19	M, 37	39·8	41·2	0·96	14·2	2·1	382/315 = 1·20
20	M, 62	52·2	38·1	1·37	ND	ND	
21	F, 59	27·5	50·2	0·54	5·8	11·8	845/787 = 1·07
22	M, 35	40·5	50·3	0·80	4·2	7·9	1 077/806 = 1·33
23	M, 56	46·2	42·4	1·08	4·9	5·2	
24	F, 56	36·9	58·7	0·62	ND	ND	
25	M, 35	42·9	44·6	0·96	4·2	ND	
26	M, 58	31·7	55·1	0·56	3·3	4·8	844/1 039 = 0·8

Patients were listed according to their viraemia level (1 to 6, bDNA HCVRNA negative; 7 to 26, bDNA HCV RNA positive) as reported in Table 2.
ND, not determined; NC, not calculated. $CD4^+/CD8^+$ ratios were not calculated when less than 500 $CD3^+$ T cells were enumerated upon immunohistochemical analysis.

$CD8^+$ ratios ranged from 0·43 to 1·68 (median 0·92) (Fig. 1). Liver-derived lymphocytes obtained from patients with detectable bDNA viraemia contained more $CD4^+$ T lymphocytes than those from patients with no detectable bDNA viraemia (Mann–Whitney U-test: U1 = 17, $P < 0{\cdot}01$). Yet there was no correlation between the $CD4^+/CD8^+$ ratios of liver-derived lymphocytes and HCV RNA levels in group 2 (linear regression $P > 0{\cdot}05$). Likewise, there was no correlation between the percentages of $CD4^+$ cells and HCV RNA levels in group 2. According to HCV genotypes, the $CD4^+/CD8^+$ ratios ranged from 0·43 to 1·53, from 0·53 to 0·99 and from 0·48 to 1·68 in group A, group B and group C, respectively. Differences were not statistically significant.

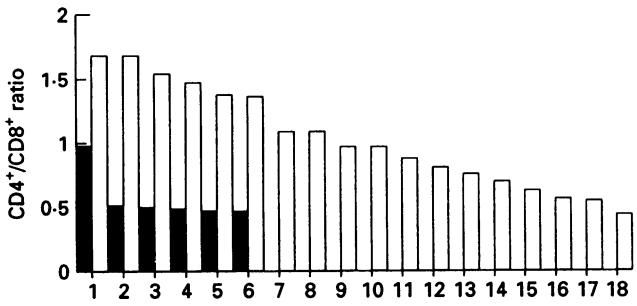

Fig. 1. Flow cytometry $CD4^+/CD8^+$ ratio of liver-derived lymphocytes in patients with chronic hepatitis C. Serum hepatitis C virus (HCV) RNA was quantified using a bDNA signal amplification assay (Quantiplex; Chiron) (Mann–Whitney U-test, $P < 0{\cdot}01$). ■, HCV RNA^-; □, HCV RNA^+.

There was no correlation between the $CD4^+/CD8^+$ ratio of liver-derived lymphocytes and aminotransferase levels.

Liver-derived lymphocytes were mostly T lymphocytes expressing the α/β TCR. According to viraemia, the percentage of T lymphocytes expressing the γ/δ TCR ranged from 3·4 to 13·27 and from 1·49 to 12·27 in group 1 and group 2, respectively. Data observed in group 1 and group 2 were not significantly different (Table 3). When comparing percentage of T lymphocytes expressing the γ/δ TCR according to HCV genotypes, the difference was not significant.

The expression of CD57 was analysed on $CD2^+$ cells. The percentage of $CD2^+CD57^+$ cells ranged from 2·43 to 11·76 and from 2·08 to 11·77 in group 1 and group 2, respectively. Data observed in group 1 and group 2 were not significantly different (Table 3). Again, when comparing the percentage of $CD2^+$ $CD57^+$ cells according to HCV genotypes, the difference was not significant.

Analysis of peripheral blood lymphocytes. The $CD4^+/CD8^+$ ratio of peripheral blood lymphocytes was determined and compared with that of liver-derived lymphocytes in seven patients. The $CD4^+/CD8^+$ ratios of peripheral blood lymphocytes ranged from 1·11 to 4·48. Those of liver-derived lymphocytes ranged from 0·48 to 1·37. There was no correlation between $CD4^+/CD8^+$ ratio of liver-derived lymphocytes and that of peripheral blood lymphocytes enumerated at the time of the biopsy for all patients ($P > 0·05$).

Immunohistochemical results. The number of $CD3^+$, $CD4^+$ or $CD8^+$ T cells was quantified in the lobules and in portal tracts and piece-meal necrosis in 15 out of the 26 patients. $CD4^+$ T cells were the most numerous cells in portal tracts and piece meal necrosis, whereas $CD8^+$ T cells were the predominant cells in lobules. The $CD4^+/CD8^+$ ratios quantified in the lobules ranged from 0·32 to 2·76 (median 0·7), whereas those quantified in portal tracts and piece meal necrosis ranged from 1·09 to 5·3 (median 2·06).

A global $CD4^+/CD8^+$ ratio was calculated from the sum of $CD4^+$ T cells and $CD8^+$ T cells observed in the three areas (Table 3). The $CD4^+/CD8^+$ ratios ranged from 0·46 to 3·13. When comparing the paired $CD4^+/CD8^+$ ratios obtained by immunohistochemistry and flow cytometry, there was no difference (Wilcoxon test, $P > 0·05$). In addition, because MoAbs used for flow cytometry and immunohistochemistry had a different origin, we compared the $CD4^+/CD8^+$ ratios obtained by flow cytometry using the different MoAbs. The results were the same, allowing the comparison of T cell analysis by flow cytometry and immunohistochemistry.

DISCUSSION

The pathogenic mechanisms of chronic hepatitis C are unknown. As the presence of mononuclear cell infiltrates is a common feature of chronic hepatitis C, intrahepatic T cell subsets were investigated for viraemia and HCV type infection. This study was performed using flow cytometry [27]. The method used for liver-derived lymphocyte analysis in this study avoided a lymphocyte isolation procedure which might lead to cell loss and bias the proportions of lymphoid subpopulation. In addition, lymphocytes were taken from needle biopsy specimens, which is easier than in most previous studies where flow cytometry was performed on resected liver tissue [28]. Recovery of mononuclear cells from liver tissue was facilitated by the absence of cirrhosis in all specimens. Using this approach, a sufficient number of liver-derived lymphocytes could be characterized phenotypically by flow cytometry. However, since immunohistochemical staining of snap-frozen liver tissue has been previously used to study lymphocyte surface markers, we compared the data obtained by flow cytometry and immunohistochemistry in 15 patients. Results obtained by flow cytometry were not statistically different from those obtained by immunohistochemical staining, but there was no statistically significant positive correlation between them. Immunohistochemistry may be limited by the variations between fields on the sections due to the heterogeneity of the liver in chronic hepatitis. In addition, the number of cells analysed may be limited. In this study, only immunohistochemical $CD4^+/CD8^+$ ratios calculated from more than 500 $CD3^+$ cells were taken into account. In contrast, flow cytometry was systematically performed on a larger number of cells (more than 2000 T lymphocytes in this study). On the other hand, cell suspension obtained from a liver specimen for flow cytometry may leave some cells in the tissue. In addition, flow cytometry does not provide information concerning the localization of the lymphocytes. The $CD4^+/CD8^+$ ratio of liver-derived lymphocytes was compared with that of peripheral blood lymphocytes enumerated at the time of liver biopsy. There was no correlation between the $CD4^+/CD8^+$ ratio of liver-derived lymphocytes and the $CD4^+/CD8^+$ ratio of peripheral blood lymphocytes. This supports our previous results [27], thereby indicating that lymphocytes we collected from the liver do not reflect blood contamination and assuming that the peripheral blood T cell population may not be representative of events occurring in liver tissue.

In the present study, the $CD4^+/CD8^+$ ratio of liver-derived lymphocytes has been shown to be higher in patients with bDNA-detectable HCV RNA than in patients without. Yet there was no correlation between the $CD4^+/CD8^+$ ratio of liver-derived lymphocytes and serum HCV RNA levels in the former group. Serum HCV RNA was quantified using a bDNA signal amplification assay. This assay, which appears to be sensitive enough for routine detection of viraemia [29], provides an estimation of the degree of viral replication. However, serum specimens with bDNA quantification values below the cutoff value do not necessarily indicate the absence of viraemia. Indeed, PCR-positive bDNA-negative serum samples suggest a low viraemia level which was seen in five out of six patients. Yet only detection of negative HCV RNA strands has been regarded as a marker of HCV replication [30]. Since serum HCV RNA cannot be quantified in our PCR-positive bDNA-negative patients, a correlation between the $CD4^+/CD8^+$ ratio of liver-derived lymphocytes and serum HCV RNA levels cannot be totally excluded. In patients with bDNA-detectable HCV RNA, the median $CD4^+/CD8^+$ ratio of 0·92 reflects an enhanced proportion of $CD2^+CD4^+$ cells as confirmed by the immunohistochemical technique. This supports results by Hata *et al.* [31], assuming that liver-derived lymphocytes obtained from patients with non-A, non-B hepatitis contained more $CD4^+$ T cells than lymphocytes recovered from the normal liver. However, no data concerning viraemia were provided in that study. $CD4^+$ T cells are commonly referred to as helper T cells. Helper $CD4^+$ T cells, acting through a variety of lymphokines they secrete, are essential for effective humoral immune responses and killing, suppression or DTH mediation [32,33]. Thus, the enhanced proportion of $CD4^+$ T cells in patients with high viral replication suggests that $CD4^+$ T cells may provide helper effects to potential cytotoxic γ/δ T cells or $CD57^+$ cells. Indeed, T lymphocytes expressing the γ/δ receptor have been shown to mediate non-MHC-restricted cytotoxicity [34]. Moreover, natural killer (NK) cells which display particular phenotypic [35] and functional characteristics are also known to mediate non-MHC-restricted cytotoxic reactions, notably against infectious agents [36]. On the other hand, $CD4^+$ T cells have been shown to display cytotoxic functions [37,38]. Taken together, these data suggest that viraemia may influence the intrahepatic T cell reactivity reflected by an imbalance in T cell subsets with regard to function via CD4 or CD8 molecules. In chronic hepatitis B, the host's immune response is assumed to play an essential role in tissue injury [39]. It has been suggested that T lymphocytes mediate liver cell necrosis [40]. Recently, we have shown that $CD4^+/CD8^+$ ratio

of liver-derived lymphocytes but not the percentage of γ/δ TCR T cells or that of $CD2^+CD57^+$ correlates with HBV replication in patients with chronic hepatitis B [27]. Therefore, it is tempting to suggest that, in comparison with chronic hepatitis B, liver cell lesions in HCV-infected patients are related to immune-mediated mechanisms. Previous studies have reported that HCV-specific T lymphocytes could be detected among liver-infiltrating lymphocytes. $CD4^+$ T cells specific for the NS4 protein of HCV were derived from the liver tissue of patients with chronic hepatitis C, and appeared to compartmentalize at the site of disease [18]. In addition, HLA class I-restricted cytotoxic T cells specific for HCV proteins were demonstrated to be located in liver tissue [17]. These data raise the question of the role of T lymphocytes in liver lesions during HCV infection. The increased proportion of $CD4^+$ T cells in the liver of the patients is intriguing, since an increased proportion of $CD8^+$ T cells would have been expected as cytotoxic effector cells. The presence of $CD4^+$ T cells might suggest a more complex immune response.

HCV displays significant sequence diversity among isolates which leads to distinct genotype identification. The differences in classification from the various laboratories have led to confusion when results are compared [41]. Thus, the influence of HCV genotype on the outcome of infection remains to be clarified [42–44]. HCV genotypes were analysed using the line probe assay, which allows fast and easy genotyping in almost all patients [23]. The French patients included in this study were mostly infected by HCV type 1b or 3a as previously reported [45]. According to HCV genotypes, neither the $CD4^+/CD8^+$ ratio of liver-derived lymphocytes nor the percentage of γ/δ TCR T or $CD2^+CD57^+$ cells were found to be significantly different in patients infected by HCV type 1b compared with those infected by HCV type 3a or those infected by other HCV types. Despite the limited number of patients, these results suggest that HCV genotype may not influence the T cell response in HCV infection. In addition, our analysis shows that neither the activity of liver disease nor serum HCV RNA levels are related to HCV genotypes, whereas the activity of liver disease is related to serum HCV RNA levels. Our results support previous studies [9,43]. The issue of genotype pathogenicity remains to be demonstrated.

In conclusion, the present study demonstrates that virus-related factors such as viraemia but not HCV genotype may influence the immune response to HCV infection. This may be reflected by the $CD4^+/CD8^+$ ratio of liver-derived lymphocytes. Further functional studies are needed to determine whether T lymphocytes are involved in the pathogenesis of HCV-induced liver disease.

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