The Cellular Infiltrate in the Liver of Patients with Fulminant Hepatitis: Analysis of Paraffin-Embedded Tissue Sections

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Intrahepatic infiltrate from 18 patients who died of fulminant hepatitis, was analyzed by an immunohistochemical method using formalin-fixed, paraffin-embedded liver sections and monoclonal antibodies. Inflammatory cells were characteristically located in the portal and periportal areas adjoining resting hepatocytes, but were infrequently found in the perivenular areas where hemorrhagic hepatocyte necrosis predominated. In the inflammatory infiltrate, T cells were the most predominant cell type, composing about two-thirds of the total hepatic infiltrate, followed by lysozyme-positive macrophages which composed about one-third of the total hepatic infiltrate, irrespective of the etiology of the fulminant hepatitis. On the other hand, B cells made up less than 2% in all cases, and plasma cells were also few, less than 2% in 12 of 18 cases. Furthermore, an enhanced display of β_2-microglobulin on hepatocyte membranes was demonstrated in all cases with remaining hepatocytes, indicating an increased expression of class I MHC antigens on these cells. These results suggest that T cells may play an important role in the pathogenesis of the portal and periportal lesions of fulminant hepatitis, probably with a help of MHC class I antigens on hepatocytes, while hemorrhagic necrosis of hepatocytes around the central veins may be caused by a different mechanism, most likely a circulatory disturbance secondary to cell-mediated immune reactions in the periportal areas.
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Key words: hepatitis, lymphocyte subset, cell-mediated cytotoxicity

Introduction

Fulminant hepatitis (FH) is a type of the most severe form of acute hepatitis resulting from submassive or massive necrosis of liver cells with severe impairment of liver functions (1). The most frequent cause is acute viral hepatitis of both B and A types. Also non-A, non-B hepatitis can become fulminant and the drug reactions comprise the next group (1). The morphology of the liver is characterized by predominant infiltration of mononuclear lymphoid cells, activation of Kupffer cells, and necrosis of liver cells (1). In chronic hepatitis, hepatic infiltrates have been well characterized *in situ* (2-4). On the other hand, only a few reports have discussed with acute hepatitis (5, 6). In the previous studies of lymphocytes obtained from biopsy cores and *in situ* characterization of lymphocytes on frozen biopsy section in acute hepatitis, it has been reported that the majority of hepatic infiltrates are T cells, and T cell-mediated immune reactions have been considered as a probable mechanism of liver cell destruction (5, 6). Recently, Mietkiewski and Scheuer (7) have characterized immunoglobulin-containing cells in acute hepatitis and found significantly more abundant IgG-containing cells in the severe form of acute hepatitis with bridging necrosis than in other forms, and claimed that B lymphocytes may play an important role in the pathogenesis of the hepatic lesions. As far as we are aware, there are few reports of *in situ* characterization of lymphocytes in the severe form of acute hepatitis with hepatic failure resulting in the patient's death (6).

Analysis of lymphocyte subsets by monoclonal antibodies is limited in many instances to frozen sections, and *in situ* localization of T and B cells in formalin fixed, routinely processed tissues has not been possible thus far.

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Recently, the commercially available monoclonal antibodies MB1 and UCHL1 have been shown to identify B and T cells respectively in paraffin sections (8, 9). This prompted us to analyze lymphocyte subsets in the routinely processed paraffin sections of the liver with massive or submassive hepatic necrosis obtained during autopsy and needle liver biopsy.

In the process of T cell-mediated cytotoxicity, expression

Table 1−(a). Clinical and Pathologic Features of 18 Cases with Fulminant Hepatitis

Case	Age/Sex	Clinical diagnoses	Main Clinical Findings						Pathological Findings			Remarks
			initial symptoms	transaminase* GOT/GPT(IU/l)	HBs/ anti-HBs	anti-HBc /HAIgM	bilirubin* (mg/dl)	disease duration** (days)	interval from initial symptom to loss of consciousness (days)	liver weight	liver pathology	associated condition	
1	58/M	fulminant hepatitis	dark urine jaundice	779/224	−/−	+/−	25.8	102	90	1100	SHN	cerebral softening	drugs: anti-coagulants
2	25/F	fulminant hepatitis	fever nausea	627/595	−/−	−/−	11.3	53	42	540	SHN	hyper-thyroidism	drug: anti-thyroid
3	36/F	fulminant hepatitis	fever	855/530	−/−	−/−	20.1	28	10	760	MHN	brain A−V malformation	blood transfusion
4	19/F	fulminant hepatitis	malaise jaundice	673/568	−/−	−/−	28.7	32	19	380	SHN	none	hospital nurse
5	31/M	fulminant hepatitis	malaise nausea	1340/843	−/−	−/−	28.4	24	16	1300	SHN	hyper-thyroidism	drug: anti-thyroid
6	60/M	fulminant hepatitis	dark urine malaise	807/252	+/−		16.3	35	16	1050	SHN	active pulmonary tuberculosis	drug: anti-tuberculous
7	51/F	fulminant hepatitis	malaise	673/258	+/−	/−	40.4	83	61	590	SHN	none	jaundice (6-yr-old)
8	66/M	fulminant hepatitis	malaise anorexia	432/475	+/−		45.0	90	86	660	SHN	none	
9	70/M	fulminant hepatitis	dark urine	531/432	−/−	/−	35.6	89	79	1250	SHN	none	jaundice (29-yr-old)
10	27/F	fulminant hepatitis	nausea	468/535	+/− ↓ −/+		16.3	43	30	780	SHN	brain hemorrhage	hospital nurse
11	69/F	fulminant hepatitis	malaise anorexia	845/748	−/−	/−	16.0	14	14	650	MHN	G−I tract hemorrhage	

*, maximum level. **, interval from initial symptoms to patient's death. ND, not determined; SHN, submassive hepatic necrosis; MHN, massive hepatic necrosis; APL, acute promyelocytic leukemia; CML, chronic myeloid leukemia.

Table 1−(b). Clinical and Pathologic Features of 18 Cases with Fulminant Hepatitis

Case	Age/Sex	Clinical diagnoses	Main Clinical Findings						Pathological Findings			Remarks
			initial symptoms	transaminase* GOT/GPT(IU/l)	HBs/ anti-HBs	anti-HBc /HAIgM	bilirubin* (mg/dl)	disease duration** (days)	interval from initial symptom to lose of consciousness (days)	liver weight	liver pathology	associated condition	
12	61/M	fulminant hepatitis	dark urine	5440/3766	−/−	/−	25.1	127	120	1280	SHN	miliary tuberculosis	gastric cancer operation (54-yr-old)
13	61/F	fulminant hepatitis	nausea vomiting	498/478	+/− ↓ −/+	−/−	16.4	101	28	1230	SHN	diabetes	myoma uteri operation (25-yr-old) drug:oral hypoglycemic agent
14	57/F	fulminant hepatitis	malaise dark urine	513/172	−/−	−/−	22.5	20	4	1120	SHN	systemic lupus erythematosus	blood transfusion drug:steroid
15	44/F	fulminant hepatitis	malaise anorexia fever	453/301	−/−	−/−	36.1	86	71	760	SHN	G-I tract hemorrhage	
16	88/F	fulminant hepatitis	malaise dark urine	1494/784	−/−	−/+	24.5	30	24	540	SHN	ischemic heart disease	
17	2/F	fulminant hepatitis	malaise	4010/1985	+/−	/−	16.0	30	18	ND	MHN	APL	in remission drugs: chemo-therapeutic agents
18	23/M	fulminant hepatitis	dark urine malaise	643/242	−/+	+/−	28.0	20	15	ND	MHN	CML	in partial remission drugs:chmo-therapeutic agents

*, maximum level. **, interval from initial symptoms to patient's death. ND, not determined; SHN, submassive hepatic necrosis; MHN, massive hepatic necrosis; APL, acute promyelocytic leukemia; CML, chronic myeloid leukemia.

of class I major histocompatibility antigen (HLA) molecules on target cells is required for efficient lysis by cytotoxic T cells (10, 11). β_2-microglobulin (β_2-m) is a small subunit or light chain of HLA class I (A, B and C) antigens (12). In the normal liver the distribution of β_2-m is quite similar to that of HLA class I antigens and neither can be detected on hepatocyte membranes (13, 14). Although HLA class I antigens are not reactive on formalin-fixed, paraffin-embedded sections, β_2-m has been found to be reactive on these materials. In the present study β_2-m was detected to speculate the expression of HLA class I antigens on hepatocytes using an immunoperoxidase method.

Materials and Methods

Liver tissues from 18 patients with massive or submassive hepatic necrosis were collected. Six had serological evidence of type B hepatitis, one had a marker of type A hepatitis, two were positive for antibody to hepatitis B surface and/or care antigen, and the remaining 9 were negative for both B and A type markers. The clinical and main pathologic findings of the cases are listed in Table 1. The liver tissues were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin (HE), Azan-Mallory, and silver impregnation.

Antibodies: The monoclonal antibodies MB1 were obtained from Bio-Science Product (Titlisstrasse, Emmembrücke, Switzerland) and UCHL1 from Dakopatts (Denmark). Although the precise nature of the antigens detected by the antibodies was not described, the manufacturers state that UCHL1 is reative with T cells and not with B cells, and MB1 reacts with B cells and only weakly with other hematopoietic cells but not with T cells. Antibodies to human lysozyme, IgG, IgM, IgA and β_2-m were purchased from Dakopatts.

Plasma cells: Plasma cells were counted according to their characteristic morphological appearance on HE-stained sections together with the help of methyl green-pyronine-stained sections.

Immunoperoxidase staining: The avidin-biotin peroxidase complex (ABC) method was performed on 5 um paraffin sections as reported previously (15). Briefly, after blocking endogenous peroxidase activity and incubation with normal serum, sections were incubated with either UCHL1 (diluted 1:100), MB1(1:5), anti-IgG (1:400), anti-IgM (1:400), anti-IgA (1:400) or anti-β_2-m antibody (1:400) for 60 min at room temperature, followed by an incubation with biotinylated second antibody for 30 min, then ABC solution (Vectastain ABC kit, Vector Lab., Burlingame, Ca., U.S.A.) for 30 min. Sections for immunoglobulin stain were treated with 0.1% trypsin (Difco Lab., Detroit, Michigan, U.S.A.) in 0.05 M Tris-HCl buffer for 20 min at room temperature before blocking endogenous peroxidase activity. Sections were treated with diaminobenzidine solution and mounted. A section for negative control in which phosphate-buffered saline instead of primary antibody was applied, was used in each set of the experiment. For positive control for UCHL1 and MB1, surgically resected lymph nodes from mesentery were used.

Counting of cells: Counting was performed at a magnification of × 400 on a Nikon microscope. After counting at least 1,000 mononuclear inflammatory cells, the percentage of positively stained cells with each antiserum was calculated.

Results

The results of analyses of inflammatory cell infiltrate in fulminant hepatitis are shown in Table 2. Although plasma cells were identified in all cases, the percentage was less than 7% of the total hepatic infiltrate in all cases. Moreover, in 12 of 18 cases the percentage was less than 2%, indicating that the plasma cells were a minor component of the hepatic infiltrate in fulminant hepatitis. There was no relationship between the percentage of plasma cells and the etiologic subtypes of fulminant hepatitis. Plasma cells were usually located in the portal area and rarely in the hepatic lobules.

UCHL1-positive cells were found most frequently in the hepatic infiltrate of fulminant hepatitis. The mean percentage was 66.8 ± 17.9, varying from 30 to 92%.

Table 2. Analysis of Mononuclear Hepatic Infiltrate in Patients with Fulminant Hepatitis

	Approximate percent of portal infiltrates						
		Cells staining with					
Case no.	Plasma cell	UCHL1	MB1	lysozyme	IgG	IgA	IgM
1	3	90	<1	7	9	6	<1
2	7	65	<1	30	18	20	12
3	<1	30	<1	63	2	10	<1
4	2	68	<1	22	18	15	7
5	6	51	<1	36	10	8	1
6	5	56	1	33	11	17	4
7	<1	56	<1	40	10	3	<1
8	1	55	2	40	1	5	1
9	2	65	<1	2	4	2	<1
10	5	51	<1	38	8	14	2
11	2	94	<1	4	3	1	<1
12	<1	49	<1	39	6	8	2
13	1	56	<1	29	4	8	3
14	<1	92	<1	4	1	1	<1
15	2	80	2	10	5	7	5
16	2	86	<1	10	4	5	<1
17	4	81	<1	24	10	11	1
18	2	78	1	7	7	10	3
Mean (%)		66.8 ±17.9		23.8 ±17.3	7.3 ±5.1	8.2 ±5.5	

UCHL1-positive cells were found not only in the portal area but also in the remaining parenchymal hepatocyte lobules (Fig. 1). They frequently showed close contact with hepatocytes (Fig. 1). The percentage of UCHL1-positive cells in the hepatic infiltrate did not differ among etiologic subtypes.

In all cases studied, MB1-positive cells totaled less than 2%. Furthermore, in 14 of 18 cases these cells totaled less than 1%, indicating that MB1-positive cells play only a minor role in the pathogenesis of fulminant hepatitis. Also the periportal proliferated bile ductules were stained with MB1 antibody (Fig. 2).

Lysozyme-positive cells varied from 4 to 63% in these cases studied, giving a mean percentage of 23.8 ± 17.3%. They were the secondary predominant cells in the hepatic infiltrate. Interestingly, in three cases with a disease duration of less than 3 weeks (cases 11, 14 and 18) and a case with the longest duration (104 days, case 1), the percentage of lysozyme-positive cells was less than 10%. Lysozyme-positive cells were preferentially located in

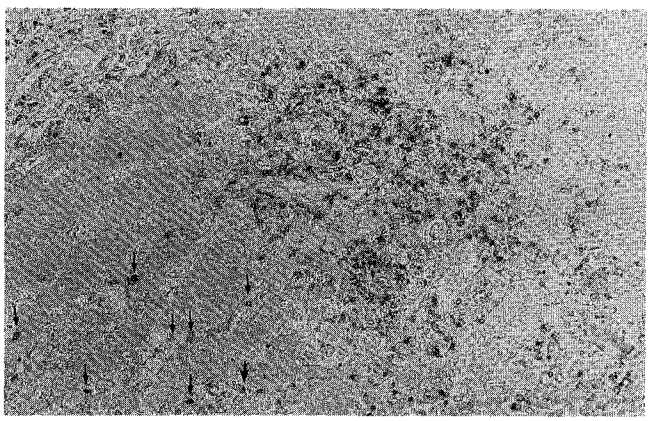

Fig. 1. Immunocytochemical staining with monoclonal antibody to T cells (UCHL1), showing dense T lymphocyte infiltrates in the periportal area adjoining the surviving hepatocytes. UCHL1-positive lymphocytes are also seen in the resting hepatocyte lobule (arrows). ABC immunostain, ×300.

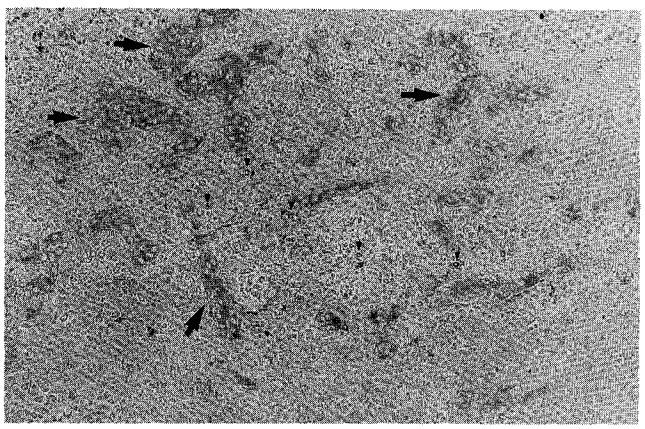

Fig. 2. Immunocytochemical staining with monoclonal antibody to B cells (MB1), showing few positive cells in the portal area (arrowheads). Proliferated bile ductules are also positive for MB1 (arrows). ABC immunostain, ×300.

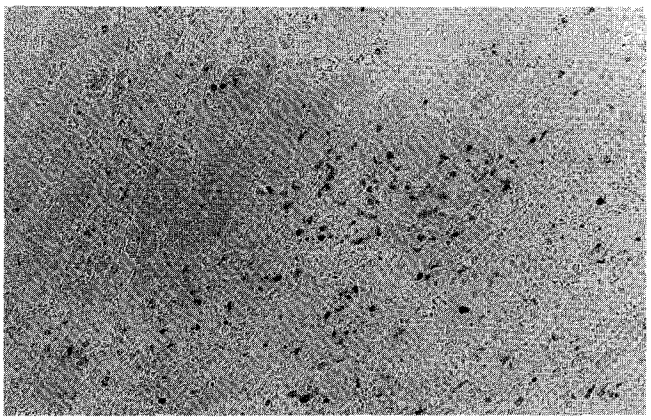

Fig. 3. Immunocytochemical staining for lysozyme-positive cells, showing positive cells in the periportal area in the vicinity of resting hepatocytes. ABC immunostain, ×300.

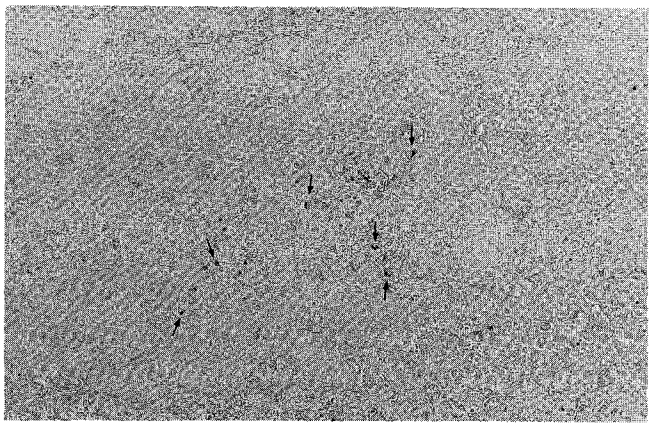

Fig. 4. Immunocytochemical staining for IgG-positive cells. Few cells stain positively (arrows). ABC immunostain, ×300.

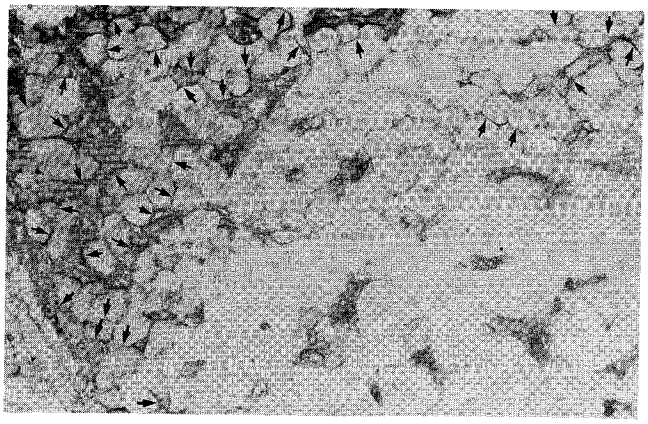

Fig. 5. Immunocytochemical staining for β_2-microglobulin. Periportal hepatocyte membranes in the vicinity of infiltrated lymphocytes stain positive for β_2-microglobulin (arrows). ABC immunostain, ×300.

the lobular periphery where massive or submassive hepatocyte necrosis occurred (Fig. 3).

IgG-positive cells varied from 1% to 18% (mean, 7.3 ± 5.1%) (Fig. 4). IgA-positive cells varied from 1% to 17% (mean, 8.2 ± 5.5%). IgM-positive cells were a minor component, varying from less than 1% to 12%, and in 9 of 18 cases the percentage was less than 1%. There was no correlation between the percentages of Ig-containing cells and the subtypes of fulminant hepatitis.

In all cases, the expression of β_2-m was demonstrated on the cytoplasmic membranes of resting hepatocytes in the vicinity of infiltrated lymphocytes (Fig. 5).

Discussion

The pathogenesis of FH is still unknown. Several hypotheses related to the pathogenesis of hepatocyte injury have been proposed based on humoral or cell-mediated immunity. Among them the most known hypotheses are those reported by Almeida and Waterson (16) and Dudley et al (17), based on humoral and cell-mediated immunity, respectively. Almeida and Waterson considered the importance of immune complexes in the induction of hepatocyte damage in hepatitis (16). In contrast, Dudley et al (17) claimed the importance of cell-mediated immunity and reported that the severity and chronicity of hepatocyte injury is determined by the ability of T cell immune responses in the host and by the amount of virus-infected hepatocytes. In the presence of normal cell-mediated immunity the severity of hepatocyte necrosis depends only on the amount of infected hepatocytes (17). There have been a number of reports to clarify the role of immune complexes or cell-mediated immunity in the pathogenesis of hepatitis. Recent evidence indicates a more important role of cell-mediated immunity than humoral immunity in the pathogenesis of hepatitis. However, the precise mechanism of hepatocyte destruction remains unknown. In hepatic histology of FH, there is a predominant infiltration of mononuclear cells in addition to the hemorrhagic hepatocyte necrosis around the centrilobular area, indicating the important role of mononuclear cells in the pathogenesis of FH. Although analysis of inflammatory cells *in situ* would be the first strategy to understand the pathogenesis of FH, only a few reports have discussed the *in situ* analysis of lymphocyte subsets (6), and the importance of cell mediated immunity in FH remains unknown.

In the present study, the predominant cells in the liver infiltrate of FH were T cells, consisting of about two-thirds of the total infiltrate, followed by macrophages consisting of about one-third of the hepatic infiltrate. B cells and plasma cells, however, were minor components of the infiltrate. Therefore, the present data indicate a probable implication of both T and macrophages, and most likely cell-mediated immunity in the pathogenesis of FH. Further analysis of T cell subsets was not performed in the present study due to the lack of monoclonal antibodies against T cell subsets which work ı paraffin-embedded tissue sections. Recently, Onji t al (6) analyzed hepatic infiltrate using frozen sections nd reported that the predominant cells in the liver are ytotoxic T cells and that these cells are frequently in ontact with hepatocytes, while helper T cells and natural iller cells are scarce and do not have contact with epatocytes, indicating the importance of cytotoxic cells in FH. Therefore, it is reasonable to consider that ıe majority of T cells detected in the present paraffin-embedded liver sections would be cytotoxic T cells. T cells are characteristically located in the portal and periportal areas adjoining surviving hepatocytes, and are scattered in the hepatic lobules in FH. On the other hand, in the centrilobular areas inflammatory cell infiltrate is scarce and hemorrhagic hepatocyte necrosis predominates, indicating that the pathogenetic processes may be different between the portal-periportal area and the centrilobular area. Predominant T cell infiltrate into the portal and periportal areas is a common finding of active hepatitis, and is reported in chronic active hepatitis and in primary biliary cirrhosis (2, 3, 18). Therefore, predominant T cells in the hepatic infiltrate is not specific to FH. However, T cells in chronic active hepatitis and primary biliary cirrhosis are considered to be responsible for the pathogenesis of hepatocyte damage, most likely via cell-mediated immune responses (2, 3, 18).

It is well known that the interaction of cytotoxic T cells with target cells requires recognition of a polymorphic determinant of class I MHC molecules on the surface of target cells (10, 11). Two mechanisms for this interaction are postualted. HLA class I antigens interact with foreign antigens to form neoantigens, and these could be recognized by a single receptor of T cells. Alternatively, cytotoxic T cells may recognize their targets by means of two independently coded receptors, one of which reacts with specific target antigens, while the other reacts with HLA class I antigens. In either case, display of HLA class I antigens is a prerequisite for T cell-mediated cell lysis.

β_2-m is closely associated with all MHC class I antigens (12). The close similarity of the distribution of MHC class I antigens and β_2-m suggests that demonstration of the latter is a useful method for evaluating changes in the distribution of the HLA class I antigens (13, 14). Although MHC class I antigens can not be demonstrated in paraffin sections, β_2-m is demonstrable in them. Therefore, we are able to evaluate changes of the expression of HLA class I antigens indirectly on hepatocytes in FH. To our knowledge, whether or not MHC class I antigens are expressed on hepatocytes of FH has not been studied so far. In the normal liver, β_2-m has been demonstrated on sinusoidal lining cells and endothelial cells but not on hepatocyte membranes (14). However, in all cases of FH, resting hepatocytes, es-

pecially those adjoining infiltrated lymphocytes, clearly expressed β_2-m on their cell surface membranes. The increased expression of β_2-m on hepatocytes may reflect an increased display of HLA class I antigens on hepatocytes and would favor T cell-mediated hepatocytolysis.

It is interesting that patterns of T lymphocyte infiltration and β_2-m display are not different among etiologic subtypes of FH. These findings imply that T cells play an important role in the pathogenesis of FH, irrespective of etiology. It is well established that host immune responses are responsible for the induction of hepatocyte damage in hepatitis B virus (HBV) infection, because of the absence of a cytopathic effect of HBV. And the same pathogenesis has been considered in non-A, non-B hepatitis infection. Furthermore, the recent findings of hepatitis A virus (HAV) also indicate that host immune responses, especially cell-mediated immunity, seem more responsible for the induction of hepatocyte damage than the cytopathic effect of HAV itself (19, 20). At any rate, the present data support the concept that cell-mediated immunity may be involved in the pathogenesis of portal or periportal changes of FH.

Lymphocyte infiltrate is scarce in the perivenular area, where hemorrhagic necrosis usually predominates in FH. This may be related to circulatory disturbance, most likely caused by molecular mediators released by lymphocytes or macrophages after immune reactions which lead to cell necrosis or disturbance of the blood circulatory system (21, 22). Therefore, morphologic changes of FH may be considered a result of a variety of host responses against causative agents, where the host cell-mediated immune responses may play an important, most probably initiative role in the induction of FH.

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