Virchows Archiv (1994) 424:471–476

ORIGINAL ARTICLE

Hiromi Himeno · Hideaki Enzan · Toshiji Saibara
Saburo Onishi · Yasutake Yamamoto

Immunoelectron microscopic observations on Leu-7 positive cells in virus-related chronic liver diseases

Received: 24 November 1993 / Accepted: 11 March 1994

Abstract We investigated the liver biopsies of 78 patients with hepatitis virus-related chronic liver diseases (B type; 14 patients, C type; 64 patients) by immunoelectron microscopy with the Leu-7 monoclonal antibody in order to determine the association of NK/K cells in virus-related chronic liver diseases. Most Leu-7 positive cells in the liver had the Pit cell morphology but a few Pit cells were Leu-7 negative. A few Leu-7 positive cells had neither Pit cell nor typical T cell morphology. No ultrastructural difference was observed in Leu-7 positive cells between hepatitis B virus- and hepatitis C virus-related chronic liver diseases. Regardless of virus type and hepatitis activity, the fine morphology of extravascular Leu-7 positive cells differed considerably from intravascular cells. Leu-7 positive cells were regularly seen in the cellular infiltrates but the ratio of Leu-7 positive cells/whole infiltrates was low. There was no correlation between the inflammatory activity of the disease and the level of Leu-7 positive cell infiltration. A virus aetiology (hepatitis-C or hepatitis-B) did not affect Leu-7 positive cell infiltration. We conclude that NK cells play only a small role in the pathogenesis of hepatitis B virus or hepatitis C virus-related hepatocytolysis, during the chronic stage.

Key words Natural killer cells · Pit cells · Large granular lymphocytes · Immunoelectron microscopy · Leu-7

Introduction

Natural killer cells (NK cells) have been defined as cells capable of mediating spontaneous in vitro cytotoxicity against a variety of target cells without deliberate prior sensitization. They have been implicated in many immunological functions, including cytotoxicity against virally transfected cells, tumour immunity and immune regulation through lymphokine secretion [21, 22, 26].

Although the contribution of NK cells to the pathogenesis of virus-related chronic liver diseases is believed to be minor, the percentage of NK cells in the cellular infiltrates varies in reports [6, 8, 9, 10, 20, 23, 25, 27, 29]. Ultrastructural detail of the NK cells infiltrating in virus-related chronic liver diseases are not available and previous investigators did not examine a sufficient number of specimens or the liver biopsies of patients with hepatitis-C virus (HCV) related chronic liver diseases.

The Leu-7 (HNK-1, CD57) antibody was originally regarded as NK cell specific [1] but recent data [22, 26] argues against the specificity of this marker. Consequently we identified NK cells on the basis of their phenotypic and morphological characteristics as large granular lymphocyte (LGL) [13, 17]. Using the prembedding method with vibratome sections [11, 12], we observed the fine subcellular structure of the Leu-7 positive cells with the same resolution as in ordinary electron micrography. We compared the findings with those in Pit cells (liver-associated NK cells), and examined their distribution and fine structure in the livers of patients with hepatitis B virus (HBV) or HCV-related chronic liver disease.

Patients and methods

Seventy-eight patients (52 men and 26 women, mean age 50.7, range 21–72 years old) with HBV or HCV-related chronic liver disease (12 chronic hepatitis-type B patients, 57 chronic hepatitis-type C patients, 2 liver cirrhosis-type B patients, 7 liver cirrhosis-type C patients) were studied. Hepatitis B surface antigen was assayed with Aeusria II 125 (Abbott Laboratories, Chicago, Ill., USA) and anti-HCV was assayed with Abbott second generation HCV EIA (Abbott). A percutaneous liver biopsy, with a Surecut needle (TSK Laboratory, Japan), was performed for diagnostic and treatment purposes. Liver tissue surrounding three (a) hemangiomas served as controls, resected from patients without other liver diseases.

The liver specimens obtained were divided into 2 parts: one was fixed in 10% neutral formalin and embedded in paraffin for

H. Himeno (✉) · T. Saibara · S. Onishi · Y. Yamamoto
First Department of Internal Medicine, Kochi Medical School, Nankoku, Kochi 783, Japan

H. Enzan
First Department of Pathology, Kochi Medical School, Nankoku, Kochi 783, Japan

light microscopy, while the other part was fixed with periodate-lysine paraformaldehyde (PLP), for 2 h at 4° C and after washing in phosphate buffer (PB), post-fixed with 1.5% osmium tetroxide in buffer pH 7.2 for 8 h. Paraffin sections, 4 μm thick were stained with haematoxylin and eosin. The histological diagnosis was made according to the standard criteria by the International Groups [7].

For immunoelectron microscopy, we obtained 40 μm-thick sections of the blocks on an Oxford vibratome and washed them 3 times in cold PBs. An avidin biotin complex (ABC) system was employed to investigate Leu-7 expression. After the 3,3′-diaminobenzidine tetrachloride (DAB) reaction, the sections were rinsed in 0.1 M PB, pH 7.2, post-fixed in 1.5% osmium tetroxide in the buffer for 1 h at 4° C and cut with a razor blade into suitable sizes. After dehydration in graded ethanols and propylene oxide, the blocks were embedded in Epon 812 epoxy resin. The ultrathin sections were stained with uranyl acetate and lead citrate, and examined in an electron microscope, JEM-100S (JEOL Ltd., Tokyo, Japan). We examined more than 20 lymphocytes for each case.

The ABC method was performed with a kit configuration (Vectastain, ABC kit for mouse; Vecter Laboratories, Burlingame, Calif., USA) and each reagent was prepared according to the manufacturer's specifications. PLP-fixed vibratome sections were incubated in 1:100 dilution of normal sheep serum for 20 min at room temperature to reduce non-specific background staining due to second antibody binding and then incubated in PBS containing a 1:100 dilution of the Leu-7 antibody (Becton Dickinson, Mountain View, Calif., USA) for 1 h at 4° C and rinsed in PBS. The sections were exposed to 1:100 dilution of biotinylated second antibody for 1 h at 4° C and then rinsed in PBS. The vibratome sections were prefixed in 0.5% glutaraldehyde in 0.1 M PB, pH 7.2, for 20 min at 4° C. After washing in cold PBS, the sections were incubated in the avidin-biotin-horseradish peroxidase (HRP) complex solution for 45 min at 4° C and washed in 0.1 M TRIS HCl buffer, pH 7.6. HRP reaction product was then revealed by incubating the sections in the 0.05 M TRIS HCl buffer with 0.05% DAB and 0.1% hydrogen peroxide for 3 min at room temperature.

As a negative control, we incubated the sections in normal mouse serum, instead of the primary antibody. As a positive control we used normal spleen specimens.

The immunostained cells were counted at × 400 magnification in the portal areas and lobules in the semi-thin sections, and the percentage of the total number of lymphocytes (200) was calculated. The results were expressed as mean values ±SD. Statistical analysis was performed with Kruskal-Wallis 1-Way Anova (analysis of variance).

Results

The histological stages of the subjects are summarized in Table 1.

In all diseased specimens, Leu-7 positive cells were seen in the sinusoids (Fig. 1A) and in the cellular infiltrates in the Glisson's sheath (Fig. 1B). But they were not predominant in the infiltrates of areas of focal necrosis or piecemeal necrosis. Table 1 shows the ratio of Leu-7 positive cells to the cellular infiltrates; this did not correlate with the histopathological activity (P=0.3620) nor with virus types (P=0.9169). The number of Leu-7 positive cells was increased in all specimens, compared with that in normal livers, which showed almost no infiltrates in the sinusoids or the Glisson's sheath.

The ultrastructure of the liver tissue was well preserved. Leu-7 positive cells had the immunoreaction product on the whole cell surface, but intensity differed to some extent among specimens and in the same specimen.

In HCV-related live specimens the most numerous constituents of Leu-7 positive cells were Pit cells, (Figs. 2–5) which were located in the sinusoids, adhering to endothelial cells, and rarely in the space of Disse. Pit cells contained electron dense granules (0.29–0.60 μm in diameter) and frequently rod-cored vesicles or empty vesicles (0.08–0.20 μm in diameter). In one cell section, 0.83±0.82 rod-cored vesicle (calculated from 200 cell sections) were seen. The nuclei were slightly indented

Table 1 Percentages of Leu-7 positive cells in the liver (*CPH* chronic persistent hepatitis; *CAH-2A* chronic aggressive hepatitis, moderate activity; *CAH-2B* chronic aggressive hepatitis, marked activity; *LC* liver cirrhosis)

	Viral aetiology	
	B type	C type
CPH	5.8±1.8 (n=2)	(n=0)
CAH-2A	10.5±7.5 (n=8)	8.6±4.0 (n=48)
CAH-2B	10.0±9.9 (n=2)	7.1±5.0 (n=9)
LC	7.0±2.1 (n=2)	7.0±5.7 (n=7)

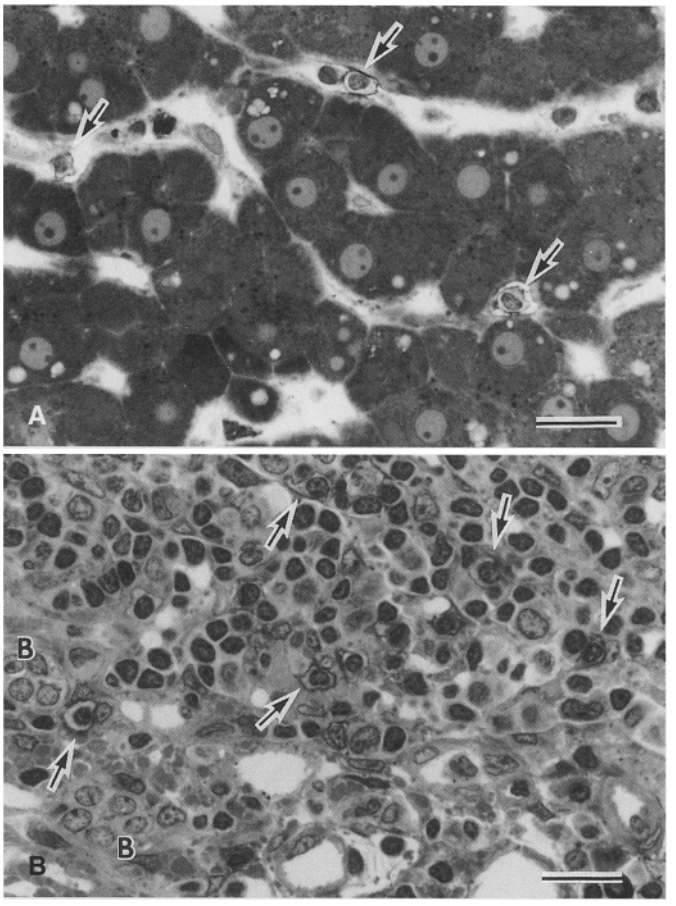

Fig. 1A Immunoperoxidase staining for Leu-7 in a biopsy liver specimen of case 24 (N.S.) on semi-thin sections. A 43-year-old man with CAH-2A, with HBs ag positive. In the sinusoid, three Leu-7 positive cells *(arrows)* were observed. *Bar* represents 20 μm. (original magnification: × 550). **B** Immunoperoxidase staining for Leu-7 in a biopsy liver specimen of case 6 (K.Y.) on semi-thin sections. A 69-year-old woman who had active LC, with HCV ab positive. In the portal area, Leu-7 positive cells *(arrows)* were observed frequently in this case only. *B*, Bile duct, *Bar* represents 20 μm. (original magnification: × 550)

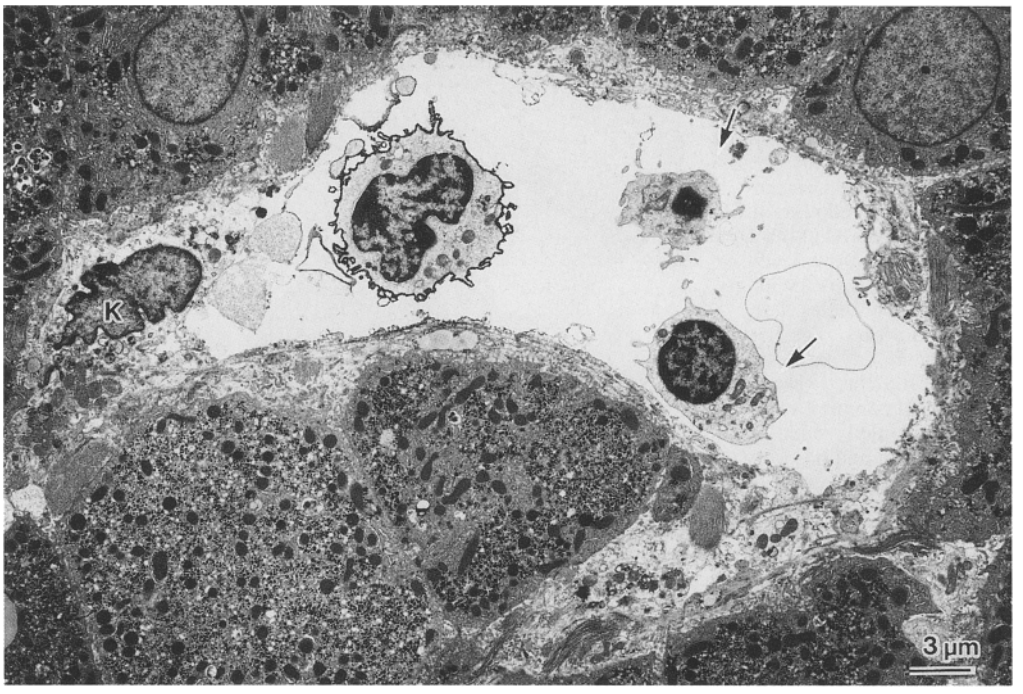

Fig. 2 Immunoelectron peroxidase staining for Leu-7 in a biopsy liver specimen of case 38 (H.M.). A 41-year-old man who had CAH-2B, HCV ab positive. On admission there was mildly elevated serum transaminase (serum GPT=77$_{IU/L}$, serum GOT=42$_{IU/L}$) and 14% of ICG$_{R15}$. In the sinusoid, a Leu-7 positive cell and two Leu-7 negative cells *(arrows)* are seen. The former is larger and has a lower nuclear/cytoplasmic (N/C) ratio, more microvilli and much convoluted nucleus than the latter. *K,* Kupffer cell (original magnification: × 3,700).

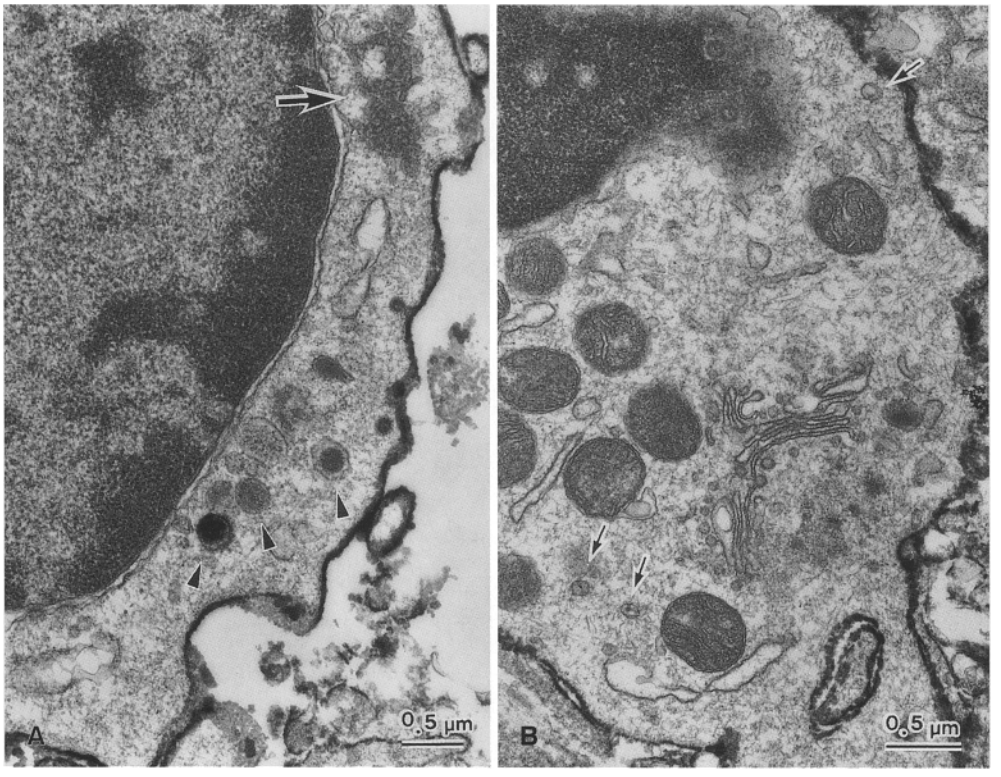

Fig. 3A Immunoelectron peroxidase staining for Leu-7 in a biopsy liver specimen of case 38 [CAH-2A-typeC]. Leu-7 positive cells rarely possessed multivesiclar bodies *(arrow)* but commonly showed characteristic electron dense granules *(arrowheads).* (In the sinusoid, original magnification: × 21,000). **B** Immunoelectron peroxidase staining for Leu-7 in a biopsy liver specimen of case 36 (O.K.). A 42-year-old man who was admitted to our hospital for liver biopsy to estimate the necessity of interferon treatment. On admission: GPT 60$_{IU/L}$, GOT 48$_{IU/L}$, HCV ab positive, ICG$_{R15}$ 9.5%. Histological examination revealed CAH-2A. Three typical rod-cored vesicles *(arrows).* Although their diameters were relatively small, a well-developed Golgi complex and abundant mitochondria were seen in a juxtanuclear portion of the Leu-7 positive cell. (In the sinusoid, original magnification: × 24,000)

and eccentrically positioned. The Golgi complexes were well-developed. Other prominent features were multivesicular bodies and parallel tubular arrays in the cytoplasm.

In extrasinusoidal area (Fig. 5), Leu-7 positive cells lost their microvilli and had pseudopodia which infrequently contacted hepatocytes or Kupffer cells. The number of intracellular organelles and characteristic granules decreased. Some Leu-7 positive cells did not have typical Pit cells morphology. Ultrastructurally typical T and B cells were negative for Leu-7.

A few Pit cells were negative for Leu-7 and Fig. 4 shows the morphological difference between Leu-7 positive and negative Pit cells.

Hepatocytes, endothelial cells, Kupffer cells and Ito cells were negative for Leu-7.

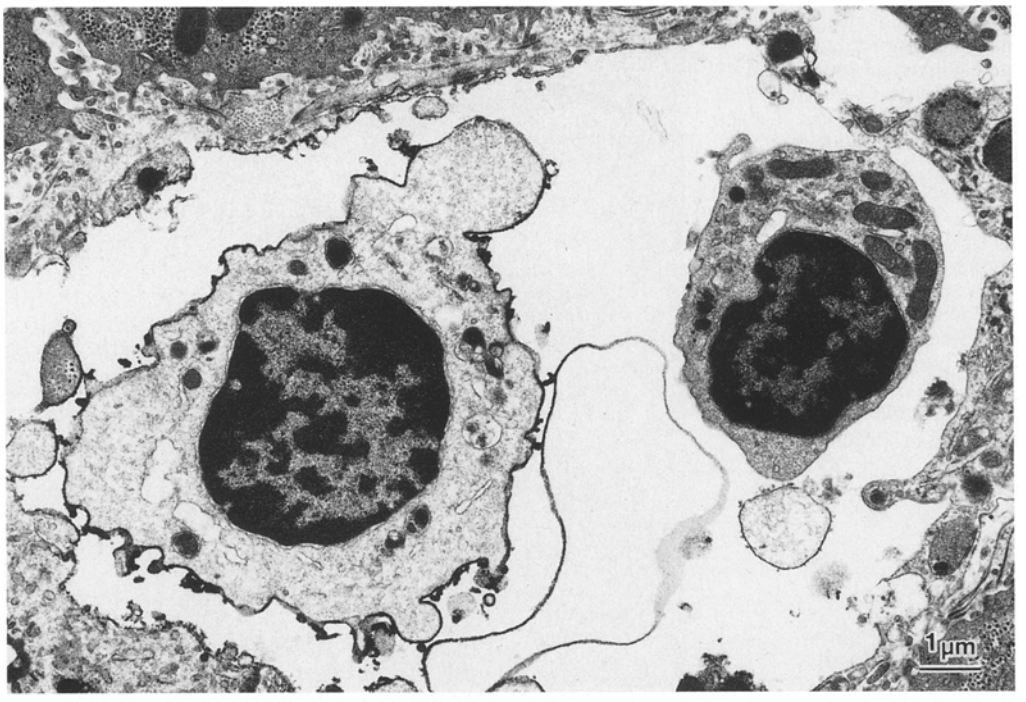

Fig. 4 Immunoelectron peroxidase staining for Leu-7 in a biopsy liver specimen of case 23 (M.N.) A 51-year-old woman HCV antibody positive 1 year ago and had slightly elevated serum transaminase levels (serum GPT=$79_{IU/L}$, serum GOT=$78_{IU/L}$) on admission. Her liver tissue showed CAH-2A at the light microscopic level. A cell stained for Leu-7 with electron dense granules in the cytoplasm is seen. The Leu-7 negative cell, has characteristic granules, rod-cored vesicles, a small Golgi complex and slightly indented nucleus. This cell is smaller and has less microvilli and a higher N/C ratio. (In the sinusoid, original magnification: × 10,000)

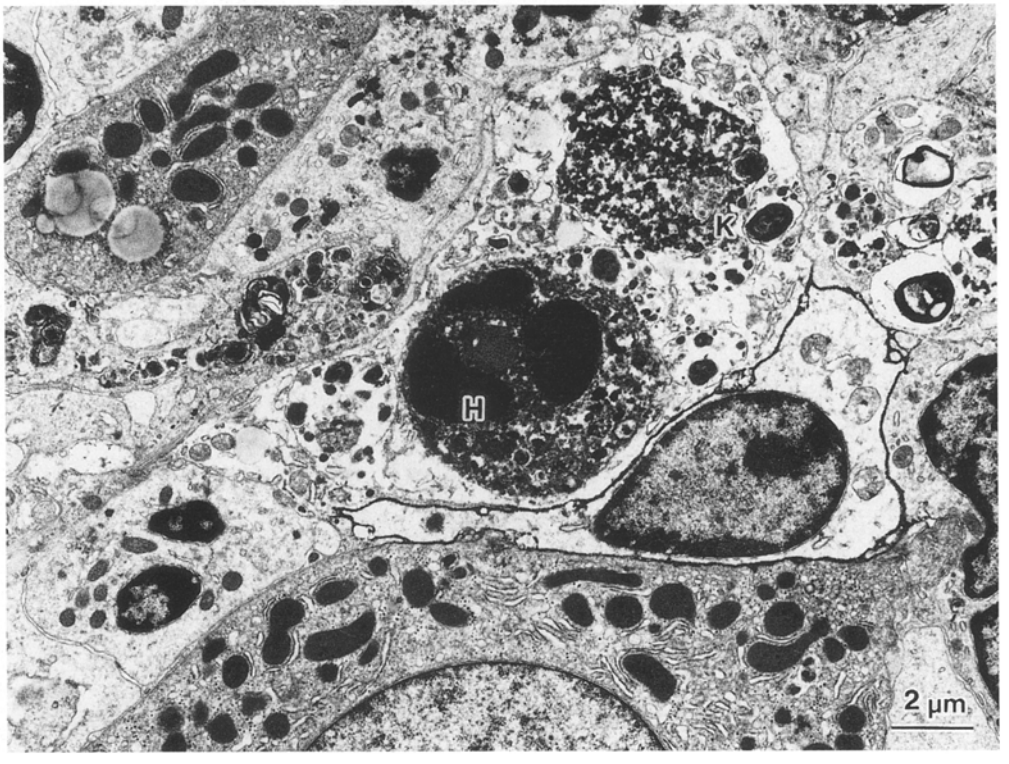

Fig. 5 Immunoelectron peroxidase staining for Leu-7 in a biopsy liver specimen of case 36 [CAH-2A-typeC]. A Leu-7 positive cell is in close contact with a Kuppfer cell (K) engulfing an apoptotic hepatocyte (H). This is an uncommon observation. The Leu-7 positive cell is in broad contact with hepatocytes. (Extrasinusoidal, original magnification: × 6,400)

Leu-7 positive Pit cells in Glisson's sheath were more pleomorphic than those in the sinusoids. Their elongated cytoplasmic projections portrayed their active amoeboid movement in the extravascular area. Mitosis of Pit cells was not detected. Biliary epithelial cells, smooth muscle cells, fibroblasts, endothelial cells and pericytes were negative for Leu-7.

Electron microscopic characteristics of Leu-7 positive cells were identical among CPH, CAH-2A, –2B and LC patients.

In HBV-related specimens, observations in the liver lobule or pseudolobule and in Glisson's sheath (Fig. 6) revealed no ultrastructural difference in Leu-7 positive cells from those of HCV-related liver specimens.

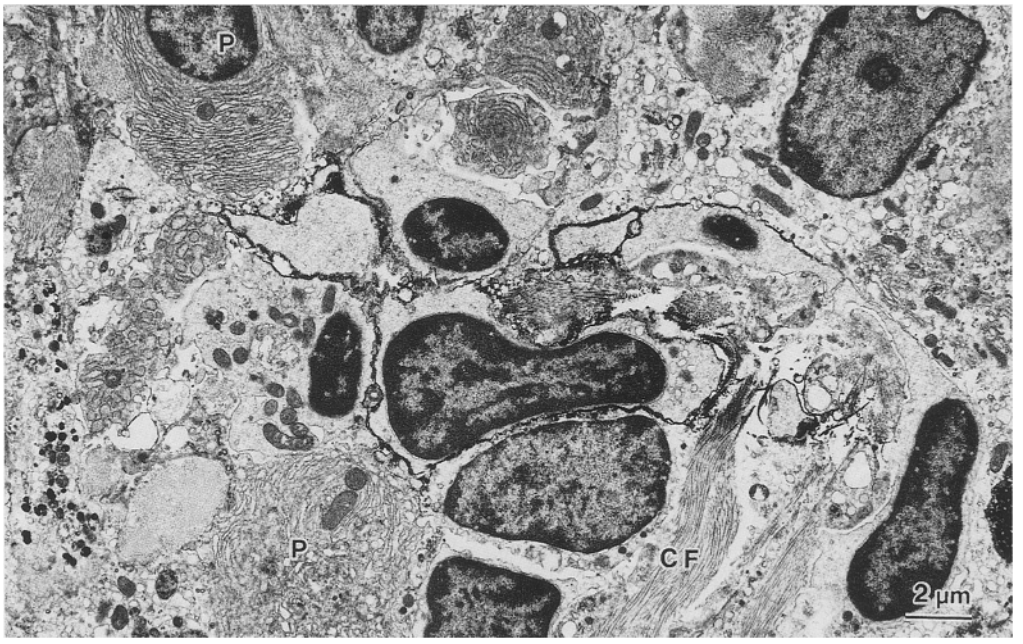

Fig. 6 Immunoelectron peroxidase staining for Leu-7 in a biopsy liver specimen of case 3 (C.I.). A 41-year-old woman who had cirrhosis, with HBs ag positive, HCV ab negative, T-Bil $1.3_{mg/dl}$, GPT $88_{IU/L}$, GOT $72_{IU/L}$ and ICG_{R15} 33%. In the cellular infiltrate in Glisson's sheath, Leu-7 positive cells were rare and did not aggregate. The Leu-7 positive bleb-like structures probably represented sectional profiles of cytoplasmic protrusions. *P*, plasma cell, *CF*, collagen fibre. (In the extravascular area, original magnification: × 5,500)

Discussion

NK cells comprise 10–15% of human peripheral blood lymphocytes and most have the LGL morphology [22]. LGLs are distributed in non-lymphoid organs, mainly the liver and lung, and in the liver, are called Pit cells, from their distinctive morphology (large lymphoid cells with dense granules, cell polarity, pseudopodia and rod-cored vesicles) [14, 15].

Rod-cored vesicles, first described in rats, were exclusively found in LGLs and were believed to be exocytosed [28]. Our observations failed to reveal evidence of exocytosis; the possibility that rod-cored vesicles are secretory vesicles derived from the Golgi apparatus is not supported by this work.

In Man, less than 1 rod-cored vesicle is seen per one cell section, while in rats, 4 rod-cored vesicles and 8 empty vesicles are present [14]. Our study confirmed the infrequency of rod-cored vesicles and empty vesicles in human diseased livers. Some Pit cells, however, were identified solely by rod-cored vesicles. Hence, this finding is a good marker to identify Pit cells at the ultrastructural level even in Man. We occasionally identified relatively small rod-cored vesicles (Fig. 3B), but their relationship to chronic diseased settings or to viral conditions is ambiguous.

Liver-associated LGLs sometimes undergo mitosis in the normal rat [28] and in rats treated with biological response modifiers [3]. The fact that we rarely detected mitosis of Pit cells indicates that numerical increase of Pit cells probably origines from extrahepatic influx rather than local proliferation in virus-related chronic liver diseases.

LGLs less frequently migrated into the space of Disse and contacted hepatocytes in our study than was reported in autoimmune hepatitis [16]. A different mechanism of hepatocytolysis might be implied in virus and autoimmune hepatitis.

CD56 (NKH-1, Leu-19), CD 16 (FcγRIII) and CD57 (HNK-1, Leu-7) are well-known markers for NKcells. No single antigen unambiguously identifies all human NK cells, however, the CD 57 antigen is expressed by about half of the peripheral blood NK cells, and only half of the CD 57 positive cells are actually NK cells [21, 22]. The difference in previous reports of the percentage of NK cells in inflammatory infiltrates in hepatitis [6, 8, 9, 10, 20, 23, 25, 27, 29], may largely originate from the specificity of antibodies used. A common observation that more NK cells infiltrate in acute hepatitis than in chronic [8] suggests separate mechanisms of hepatocytolysis in the course of time.

Numerous investigators have demonstrated that NK cells can lyse virus-infected target cells in vitro selectively [22]. Murine hepatitis virus infections are more severe in mice treated in vivo with anti-asialoGM1 (which was reported to inhibit NK function) [4]. In Man, a patient with a marked susceptibility to herpes viruses has been reported to show an extreme deficiency of NK cells [2]. This suggesting that the capability of NK cells is mostly confined to acute phase anti-viral activity. Pit cells in the liver may act as an early phase resistance mechanism to virus infections, whereas in chronic stages, as shown here in virus-related chronic liver diseases, their contribution is minor; other responses may dominate. In HBV infection, cytotoxic T lymphocytes (CTL) are thought to be responsible for the pathogenesis of chronic type B hepatitis [18, 19]. Recently in HCV infection, CD8+CTL that recognized an epitope from the HCV sequence were reported [24].

In cirrhotic patients, NK cell activity has been reported to be significantly decreased [5]. In our observations, Pit cell morphology and number did not differ among

CH and cirrhotic patients. This is partly because morphology and number do not correlate with functional activity and partly because of the difference in peripheral and liver-specific NK.

In sum, regardless of virus type (HCV or HBV) and histological stage, the ultrastructure and quantity of Leu-7 positive cells in the liver are similar. Prominent electron microscopic morphological differences in Leu-7 positive cells depends on their location; whether they are extravascular or intravascular. We conclude that NK cells are not a major factor in pathogenesis in HCV or HBV related liver disease.

Acknowledgements We wish to thank Mr. M. Shirota and Mr. K. Yagyu (Medical Research Laboratory, Kochi Medical School) for technical assistance. We would also like to acknowledge Mr. Boone White for his language consultation.

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