Gastroenterologia Japonica
Vol. 18, No. 4

Printed in Japan

—Original Article—

INTRAHEPATIC DISTRIBUTION OF T CELL AND T CELL SUBSETS IN CASES WITH TYPE B CHRONIC LIVER DISEASE BY PEROXIDASE-LABELED ANTIBODY METHOD USING MONOCLONAL ANTIBODIES

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Summary

Intrahepatic distribution of T cell and T cell subsets was studied in 23 cases with type B chronic liver disease (of which 19 cases were also positive for hepatitis B e antigen) and in 6 cases with non-B chronic liver disease by indirect peroxidase-labeled antibody method using monospecific anti-T antibodies and other reagents (anti-Leu series). Hepatitis B surface antigen (HBsAg) and hepatitis B core antigen (HBcAg) in liver tissue were also observed by the peroxidase-labeled antibody method. Membranous expression of HBsAg was found in 16 out of 23 patients with sero-HBsAg, and HBcAg was detected in 15 of them. In these patients, pan-T cells (Leu-1 positive cells) were the predominant cells in the portal tract and the parenchyma. Particularly, T cytotoxic/suppressor cells (Leu-2a positive cells) were often recognized both in sites of piecemeal necrosis and focal necrosis. In some patients whose liver biopsy specimens were obtained during acute exacerbation of chronic hepatitis, T cytotoxic/suppressor cells as well as pan-T cells were increased remarkably in sites of piecemeal necrosis and focal necrosis. These results suggest that T cell cytotoxicity may play an important role in the pathogenesis of liver cell necrosis in type B chronic liver disease.

Key Words: *T cell, T cell subsets, monoclonal antibody, peroxidase-labeled antibody method, type B chronic liver disease.*

Introduction

In previous studies it was indicated that hepatitis B virus (HBV) is not directly cytopathic and the pathogenic manifestations of type B hepatitis have been attributed to the host immune response[1,2]. The role of the cellular immune response has been lately emphasized in the pathogenesis of chronic hepatitis B virus infection[3,4], but there is not enough direct evidence to suggest the cellular mechanism within the liver. The cellular responses in type B hepatitis have been suggested by the evaluation of peripheral blood lymphocytes[5], the response of peripheral blood lymphocytes to various T cell mitogens[6], and lymphocyte cytotoxicity tests toward various target cells[7-9]. Moreover, some reports suggest T cell cytotoxicity within

Received February 21, 1983. Accepted April 4, 1983.

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The author would like to thank Professor H. Nagashima and Dr. G. Yamada of the First Department of Internal Medicine, Okayama University Medical School, for valuable advice regarding this manuscript. This study was supported in part by a grant from the Japanese Ministry of Health and Welfare, and from the Japanese Ministry of Education Science and Culture (Grant No. 57570280).

the liver; abundant T cells were demonstrated by immunohistochemical techniques in suspensions or sections of liver tissue obtained from cases of chronic hepatitis B[10-12]. However, the intrahepatic distribution of T cell subsets has not been studied in detail. On the other hand, interactions between HBV infected cells and lymphocytes were observed by electron microscopy[13,14]. These results also suggest that the mechanism of the liver cell damage might be cellular responses to hepatocytes, but the target antigens expressed on the surface of hepatocytes have not been determined.

In this paper cellular type immune response against viral antigens expressed on the liver cell surface was investigated in order to clarify the mechanism of liver cell injury in chronic hepatitis B. For this purpose the intrahepatic viral expression (HBsAg and HBcAg) and the activity of both T lymphocytes and its subsets were studied in liver biopsy specimens by the peroxidase-labeled antibody method.

Materials and Methods

Liver biopsy specimens were obtained by peritoneoscopy from 23 patients who were seropositive for HBsAg by the reversed passive hemagglutination method[15] (19 patients were also positive for hepatitis B e antigen by radioimmunoassay[16]) and from 6 non-B (negative for both HBsAg and antibody to HBsAg) patients. Each biopsy specimen was divided into two portions. One was fixed in Bouin's solution for routine light microscopy, and the other was fixed in a periodate-lysin-paraformaldehyde (PLP) fixative[17] for immunohistochemical study. They were histologically diagnosed according to the review by an International Group[18]. Out of 23 HBsAg-positive cases, 4 cases were diagnosed as chronic aggressive hepatitis (CAH) 2A, 13 cases as CAH-2B, 4 cases as chronic persistent hepatitis (CPH), and remaining 2 cases as liver cirrhosis (active type). Out of 6 non-B patients, one case was diagnosed as CAH-2A, 4 cases as CAH-2B, and the remaining one case as alcoholic hepatitis. Histologically normal human lymph nodes obtained at surgery from a patient with peptic ulcer were also fixed with a PLP fixative and stained for the specificity of monoclonal antibodies.

Mouse monoclonal antibodies against human T cell antigen and its subset antigens were used as follows; antibody to human pan-T cell antigen (anti-Leu-1, Becton Dickinson), antibody to human T cytotoxic/suppressor cell antigen (anti-Leu-2a, Becton Dickinson), and antibody to human T helper/inducer cell antigen (anti-Leu-3a, Becton Dickinson). Mouse monoclonal antibody against Ia-like antigen (anti-HLA-DR, Becton Dickinson) was also prepared. The Fab′ fragment of IgG fraction of rabbit anti-mouse immunoglobulins (Dakopatts) was conjugated with horseradish peroxidase (HRPO) (Sigma Chemical Co., type VI) by the method of Nakane et al.[19]. Fab′ of each IgG isolated from antiserum to HBsAg (anti-HBs) produced in rabbits[20] and from antiserum to HBcAg (anti-HBc) raised in guinea pigs[21] was also conjugated with HRPO.

Liver biopsy specimens fixed in a PLP fixative were washed in 0.05 M sodium phosphate buffer, pH 7.2 with 8.5% sucrose for 4 hours, and embedded in Ames OCT compound. Quickly frozen sections (6 μm in thickness) were dried and washed in PBS. Periodic acid and sodium borohydride treatment[22] was performed to inactivate endogeneous peroxidase in the liver. For lymphocyte staining, the sections were incubated with each monoclonal antibody for 12 hours, and further incubated with HRPO-labeled rabbit Fab′ anti-mouse immunoglobulins for 4 hours. For the observation of HBsAg and HBcAg, the sections were incubated with HRPO-labeled Fab′ of anti-HBs and anti-HBc. Then, they were reacted

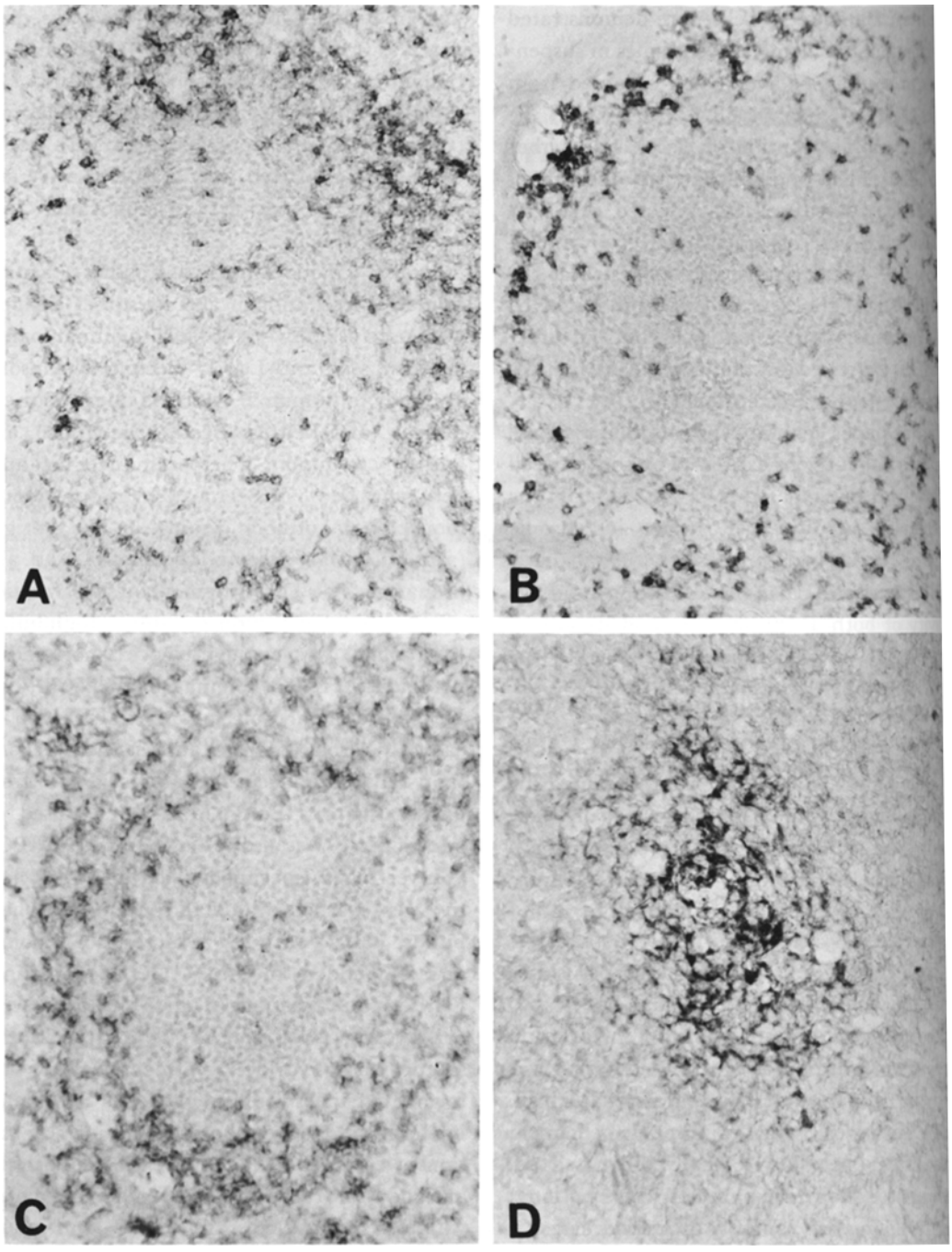

Fig. 1. Distribution of pan-T cells, T cytotoxic/suppressor cells, T helper/inducer cells, and Ia-positive cells in the human lymph node. A. Pan-T cells (Leu-1 positive cells) are mainly observed in paracortical region. B. T cytotoxic/suppressor cells (Leu-2a positive cells) are scattered in paracortical area. C. T helper/inducer cells (Leu-3a positive cells) are also demonstrated in paracortical region. D. Ia-positive cells are mainly detected in the germinal center of the follicle. (counterstained with methyl green, ×240)

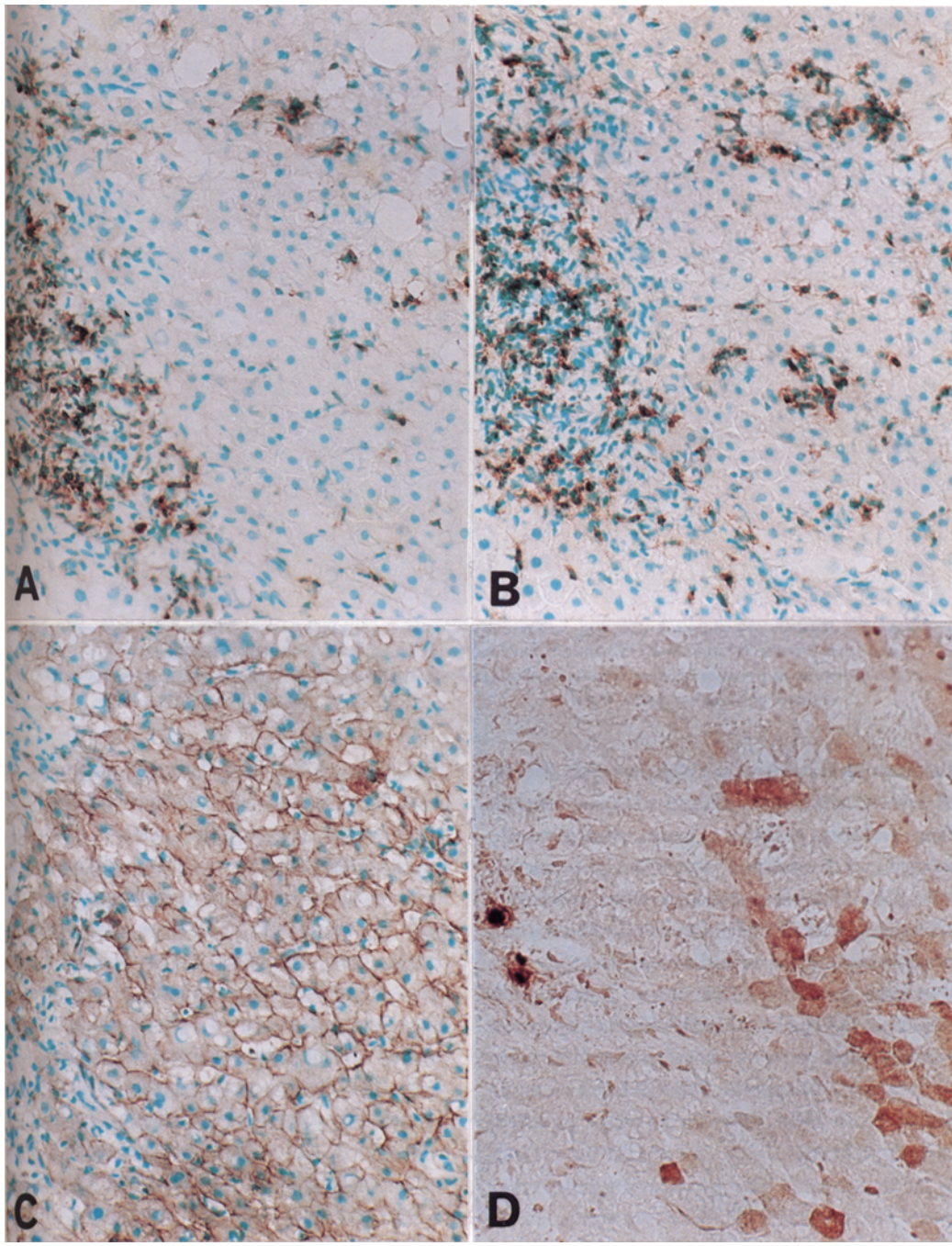

Fig. 2. Intrahepatic distribution of pan-T cells, T cytotoxic/suppressor cells, HBsAg and HBcAg in a case with CAH-2A, in a biopsy specimen was obtained during acute exacerbation. A. Majority of pan-T cells are mainly localized in the portal area and in the focal necrosis of the parenchyma. B. T cytotoxic/suppressor cells are also distributed in the parenchyma (in the sinusoid and in sites of focal necrosis) as well as in the portal tract. C. HBsAg shows honeycomb-like distribution. D. HBcAg is detected in the cytoplasm of many liver cells. (counterstained with methyl green except for D, ×240)

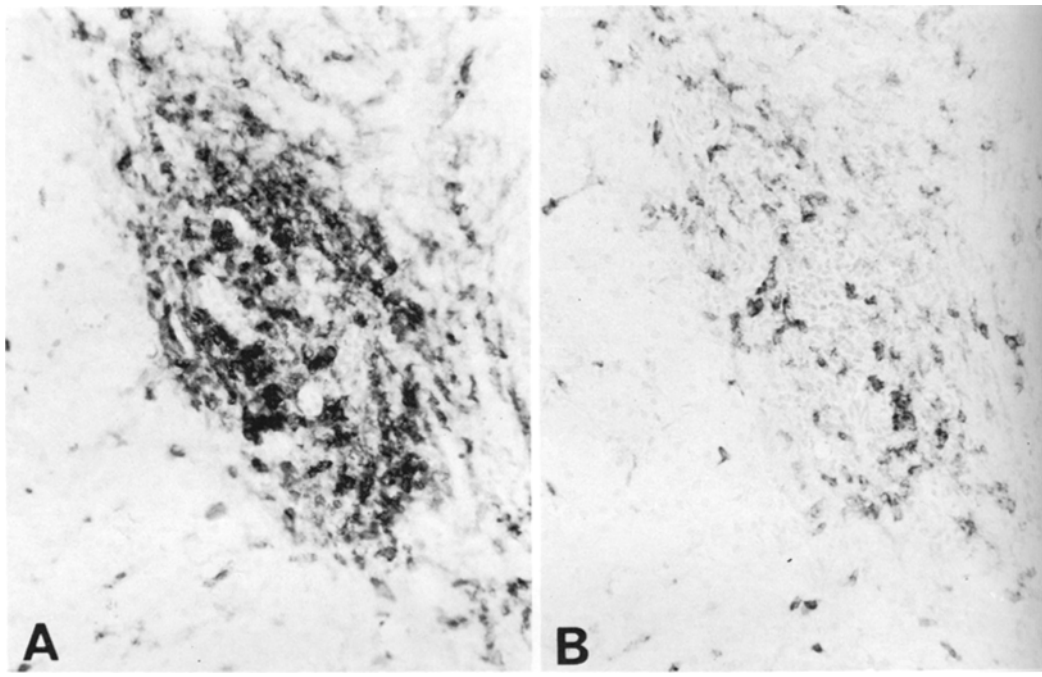

Fig. 3. Distribution of pan-T cells and T cytotoxic/suppressor cells in a patient with CAH-2B. A. Pan-T cells were demonstrated in about 80% of mononuclear cells infiltrating the portal area. B. T cytotoxic/suppressor cells are shown in about 20% of mononuclear cells of the portal area. (counterstained with methyl green, ×240)

with diaminobenzidine (DAB)-peroxide for 10 minutes. After counterstained with the methyl green solution, they were dehydrated, mounted, and observed under a light microscope. The following controls were used: (1) PBS instead of HRPO-Fab′ to allow the evaluation of endogenous peroxidase activity; (2) normal mouse sera instead of mouse monoclonal antibodies; (3) specific HRPO-Fab′ conjugates absorbed with acetone dried normal human liver powder; (4) specific HRPO-Fab′ of anti-HBs or anti-HBc absorbed with purified HBsAg or HBcAg.

Results

Distribution of T cell, T cell subsets, and Ia-positive cells were observed in the lymph nodes. Large amounts of pan-T cells (Leu-1 positive cells) were distributed in the paracortical region (**Fig. 1A**). T cytotoxic/suppressor cells (Leu-2a positive cells) were scattered in the paracortical region (**Fig. 1B**). T helper/inducer cells (Leu-3a positive cells) were also demonstrated in the paracortical region (**Fig. 1C**). Ia-positive (HLA-DR positive cells) were distributed in the germinal center of the follicle (**Fig. 1D**). No specific staining of these lymphocytes was not observed by normal mouse sera instead of mouse monoclonal antibodies.

In 10 HBsAg-positive patients with chronic hepatitis or cirrhosis, the proportion of pan-T cells and T cell subsets was examined. Pan-T cells consisted of 67.0 ± 11.8 (Mean ± S.D.)% of mononuclear cells infiltrating in the liver. Moreover, 33.9 ± 8.1% of mononuclear cells were positive for Leu-2a antigen and 37.6 ± 5.2% of them positive for Leu-3a antigen. There were few Ia-positive cells in the portal

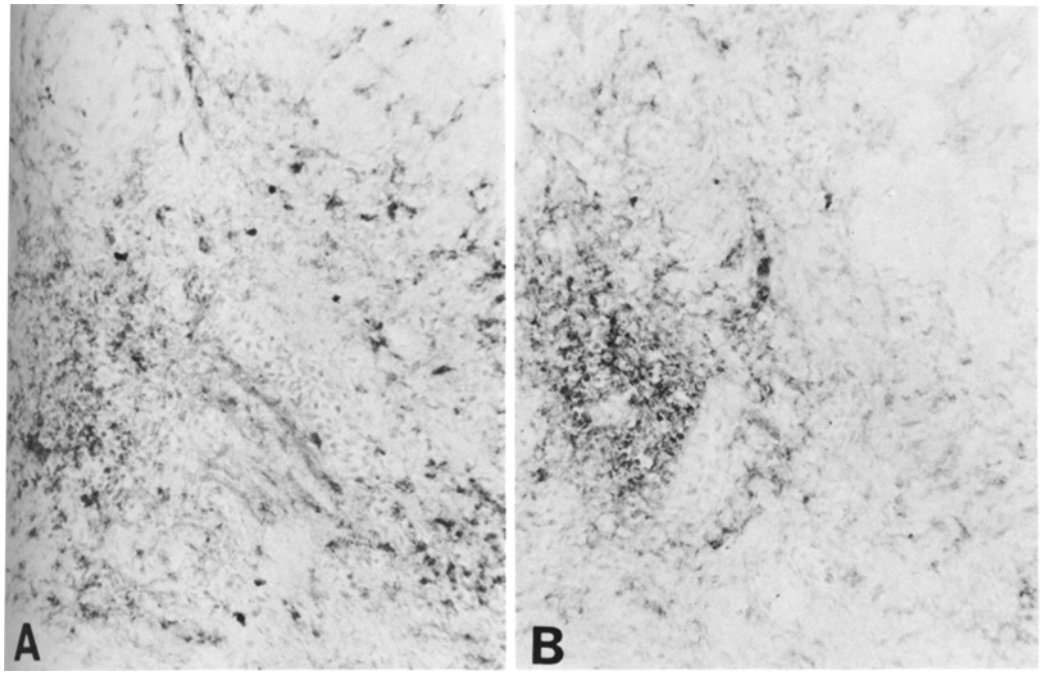

Fig. 4. Distribution of T cytotoxic/suppressor cells and T helper/inducer cells in a patient with CAH-2B. A. T cytotoxic/suppressor cells are prominent in the periportal area. B. T helper/inducer cells are mainly observed in the central area of the portal tract and rare in the periphery. (counterstained with methyl green, ×240)

area, but it was difficult to show the distribution of B cells in the parenchyma because Kuppfer cells and macrophages also seemed to be positive for Ia antigen. The majority of pan-T cells were distributed in the portal area, particularly in sites of piecemeal necrosis (**Fig. 2A, 3A, 5A**). T cytotoxic/suppressor cells and T helper/inducer cells were also mainly demonstrated in the portal tract (**Fig. 3B, 4**). T cytotoxic/suppressor cells were prominent in the periportal area, as compared with T helper/inducer cells which were more in the central part of the portal area (**Fig. 4**). However, the appearance of both pan-T cells and T cell subsets was not so remarkable in the sinusoids of the parenchyma. In 3 of 7 cases during acute exacerbation of chronic hepatitis, marked infiltration of pan-T cells and T cytotoxic/suppressor cells was observed in the sinusoids (particularly in sites of the focal necrosis) as well as in the periportal area (**Fig. 2A, 2B**). These lymphocytes were often in contact with liver cells. There were more pan-T cell and T cell subset infiltrates in the patients with CAH (**Fig. 2–5**) compared to patients with CPH (**Fig. 6**). However in one patient with CPH, many pan-T cells and T cytotoxic/suppressor cells were found in the parenchyma (**Fig. 7**). No significant difference between type B and non-B chronic hepatitis was recognized in the infiltrates of both pan-T cells and T cell subsets into the portal tracts.

Intrahepatic HBsAg was detected in 22 out of 23 patients with sero-HBsAg. A membranous HBsAg pattern was observed in 16 of them (in 14 of 19 patients with HBeAg), and intrahepatic HBcAg was demonstrated in 15 of them (in 13 of 19 patients with HBeAg). In

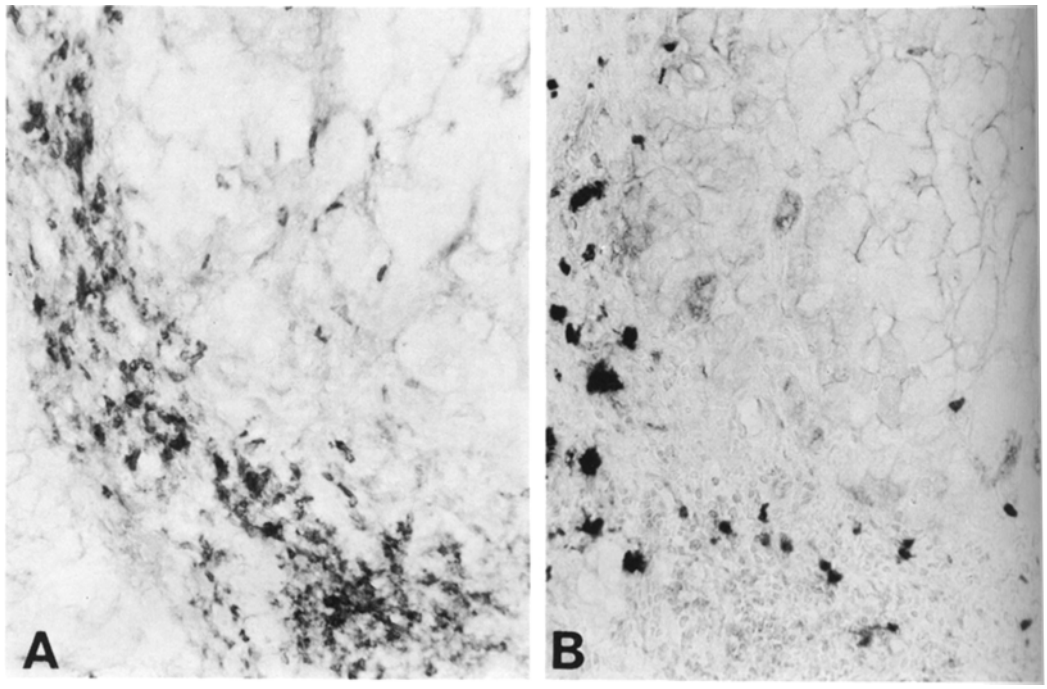

Fig. 5. Distribution of pan-T cells and HBsAg in a patient with CAH-2B. In this case, some pan-T cells (shown in A) appear in contact with HBsAg-positive hepatocytes (shown in B) in serial sections. (counterstained with methyl green, ×240)

serial sections of some patients with a honeycomb-like distribution of membranous HBsAg and a diffuse expression of cytoplasmic HBcAg, many pan-T cells and T cytotoxic/suppressor cells were observed in the sinusoids as well as in the portal tract (**Fig. 2**). These lymphocytes were often localized in sites of piecemeal necrosis and focal necrosis, and some of them were in contact with hepatocytes containing membranous HBsAg or cytoplasmic HBcAg, but there no lymphocytes were found to be present on most hepatocytes despite membranous expression of viral antigen (**Fig. 2, 5**). In patients with CPH, no apparent relation between hepatocytes with HBsAg or HBcAg and the distribution of both pan-T cells and T cytotoxic/suppressor cells were also found (**Fig. 6, 7**).

Discussion

In this paper, we have shown intrahepatic distribution of T cell and T cell subsets in cases with type B chronic liver disease by indirect peroxidase-labeled antibody method using monoclonal antibodies. Although there have already been some reports in which the intrahepatic distribution of lymphocytes subpopulation (T cells and B cells) has been investigated[10-12], the problems of specificity of the antibody used and the differences of the diseases of the patients made it difficult to evaluate. In this study, we have used monoclonal antibodies to T cell and its subset antigens (anti-Leu series). Their specificity has already been confirmed by others[23-25], and we have also made sure of their specificity by observing the distribution of T cell and its subsets in the

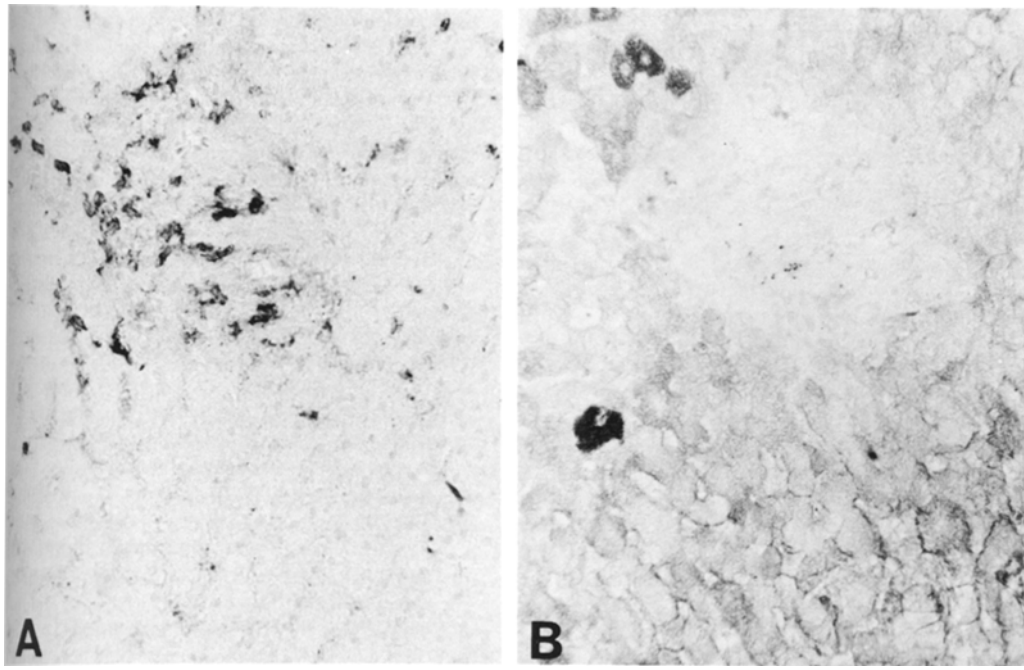

Fig. 6. Distribution of T cytotoxic/suppressor cells and HBsAg in a patient with CPH. A. Some T cytotoxic/suppressor cells are shown in the portal tract. B. HBsAg is detected both on the surface of the liver cells and in the cytoplasm. (counterstained with methyl green, ×240)

lymphnode using these antibodies.

In the present study of liver specimens in type B chronic liver disease, T cells are predominant in almost all cases and mainly distributed in the portal area. Especially they infiltrated the piecemeal necrosis of the periportal area and occasionally in focal necrosis of the parenchyma. These results are consistent with previous findings[10-12]. Moreover, we observed T cytotoxic/suppressor cells often distributing in sites of piecemeal necrosis, and occasionally in focal necrosis of the parenchyma. In these cases, T cytotoxic/suppressor cells as well as pan-T cells were occasionally in contact with liver cells in piecemeal necrosis of the periportal area and in focal necrosis of the parenchyma. These findings were more often demonstrated in liver biopsy specimens taken during acute exacerbation. Therefore, we suggest that T cell cytotoxicity may contribute to liver cell injury of type B hepatitis. On the other ahnd, Alberti et al.[26] found that membrane-bound immunoglobulin G (IgG) is present on liver cells of chronic type B hepatitis, and antibody dependent cell-mediated cytotoxicity (ADCC) may be related to the liver cell necrosis. Furthermore, there were some reports which suggested the possible involvement of natural killer (NK) cells in HBV infection[27]. These works predict some other host immune responses such as ADCC or cytotoxicity by NK cells for cell lysis in chronic HBV infection. Further immunohistochemical studies using other monoclonal antibodies will be necessary to elucidate the cellular cytotoxicity.

Several viral antigens were suggested to serve as the target antigens of the host immune response in the liver injury of type B chronic liver disease. In particular, HBsAg may be one of the candidates[7-9]. In this regard, the author

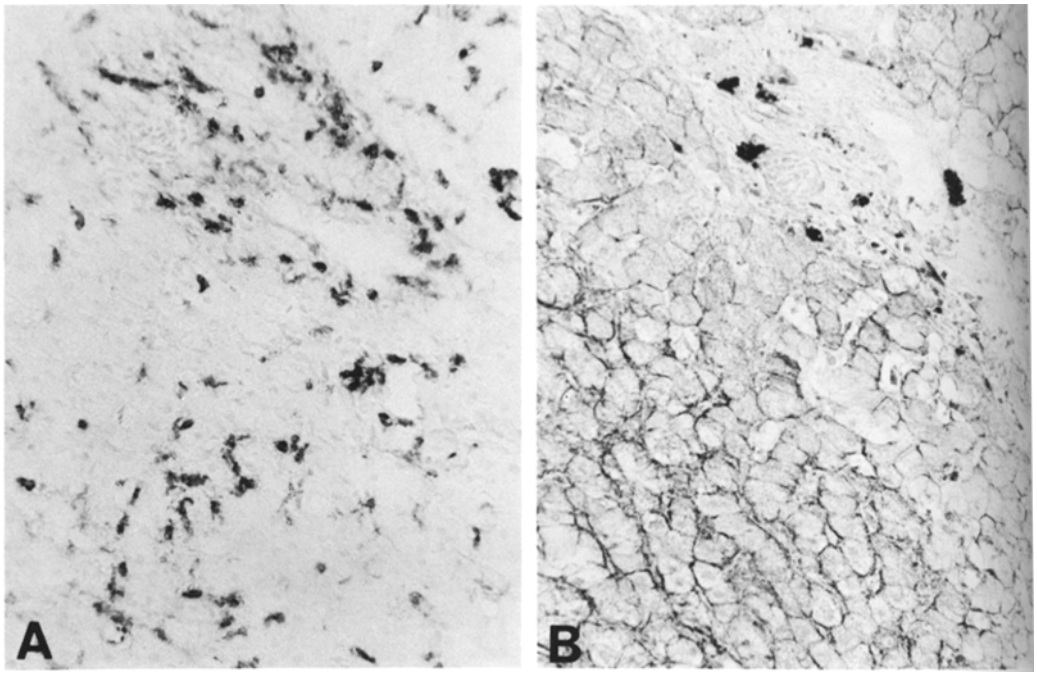

Fig. 7. Distribution of T cytotoxic/suppressor cells in another patient with CPH. A. In this case T cytotoxic/suppressor cells are found not only in the portal tract but also in the parenchyma. B. HBsAg shows a diffuse membranous pattern. (counterstained with methyl green, ×240)

and his colleagues previously reported that HBsAg is expressed on liver cell surface and may be a target antigen for cellular cytotoxicity[20,21,28-30]. In the present study, a diffuse membranous pattern of HBsAg was also observed in most cases with HBeAg. In some of these patients, many T cytotoxic/suppressor cells as well as pan-T cells were observed in the portal area and the lobular parenchyma. However, these lymphocytes are not recognized on the surface of most hepatocytes possessing membranous expression of viral antigen. This could indicate that such cytotoxicity is not directed solely against HBsAg. On the other hand, it was reported by Mondelli et al.[31] that T cell cytotoxicity in patients with chronic HBV infection was directed against determinants resembling HBcAg on the plasma membrane of hepatocytes. In contrast with their results, we could not find the significant correlations between the intrahepatic distribution of HBcAg and that of T cytotoxic/suppressor cells or that of pan-T cells. Subsequently, T cell cytotoxicity may be directed against HBV-induced neoantigens or liver membrane protein altered by HBV infection. Otherwise, these differences may be attributed to individual differences in cases or to the clinical stage. Further studies in more liver tissues and in sera are necessary for better understanding of the cellular mechanism in chronic hepatitis B.

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