Gastroenterologia Japonica
Copyright © 1990 by The Japanese Society of Gastroenterology

Vol. 25, No. 1
Printed in Japan

— Original Article —

Immunohistochemical investigation of hepatitis B virus associated antigens, HLA antigens and lymphocyte subsets in type B chronic hepatitis

Keiichi AOYAMA[1], Takashi KOJIMA[2], Kyouichi INOUE[1], and Hiroshi SASAKI[1]
[1]*Third Department of Internal Medicine, Toyama Medical and Pharmaceutical University, Toyama 930-01, Japan; and* [2]*Second Department of Pathology, Nihon University School of Medicine, Tokyo 173, Japan*

Summary: HLA antigens, hepatitis B virus (HBV)-associated antigens and lymphocyte subsets in liver tissue from 35 patients with HBs antigenemia were studied using an immunoperoxidase double staining method and immunoelectron microscopy in order to clarify the immune mechanism of hepatocyte lysis in type B hepatitis. Immune light and electron microscopy using monoclonal antibodies to lymphocyte subsets revealed that infiltrating lymphocytes in the areas of piecemeal necrosis and focal necrosis were predominantly CD8-positive, showing direct contact with hepatocytes. In contrast, CD4(+) cells were infrequently observed in necrotizing inflammatory lesions. HLA-A,B,C antigens were mainly found on hepatocytes in areas of piecemeal necrosis and focal necrosis, in association with CD8(+) lymphocyte infiltration. HLA-DR antigens were demonstrated on a few hepatocytes in the same lesions. In cases of CAH with serum HBeAg positive, HLA-A,B,C, antigens and HBV antigens simultaneously demonstrated on the same hepatocytes. Especially, hepatocytes expressing both HLA-A,B,C antigen and HBsAg on the plasma membrane showed direct contact with CD8(+) lymphocytes. This finding fullfilled the morphological requirements for HBsAg as a target antigen. On the other hand, HBcAg was hardly demonstrated in the liver cell membrane but was demonstrated mainly in the cytoplasm. Compared with the nuclear localization of HBcAg in cases of NSR, cytoplasmic localization of this antigen may be associated with membranous expression of new antigens induced by HBV infection. *Gastroenterol Jpn 1990;25:41–53*

Key words: *HBcAg; HBsAg; HLA antigens; immunoperoxidase double staining; lymphocyte subsets*

Introduction

Although the exact mechanism of hepatocyte lysis in cases of persistent HBs antigenemia has not yet been clarified, immune reactions, particularly cell-mediated immunity, have been considered likely[1-5]. Recent advances in the development of monoclonal antibodies against lymphocyte subsets have immunohistochemically explored cytotoxic T-cells as playing an important role in the pathogenesis of type B hepatitis[6-8]. For cytolysis to occur under these conditions, viral antigens and HLA antigens must be expressed on the plasma membrane of the target hepatocytes. The HLA molecules were noted to be transmembrane glycoproteins important in cell-to-cell communication as receptors[9]. It was also noted that viral infection altered the intensity of expression of HLA antigens on infected cells, and influenced the immune response to infected cells[10,11]. In this respect, hepatitis B virus (HBV) is also considered to play a causative role in HLA antigen expression on infected hepatocytes, and to moderate

Received June 6, 1989. Accepted July 28, 1989.
Address for correspondence: Keiichi Aoyama, M.D., Third Department of Internal Medicine, Toyama Medical and Pharmaceutical University, Sugitani 2630, Toyama, 930-01, Japan.

the immune reaction induced by cytotoxic lymphocytes[12].

In the present study, we analyzed the topographic relationship among HBV antigens, HLA-A,B,C and -DR antigens and lymphocyte subsets in order to clarify the immune mechanism of type B hepatitis B using immunoperoxidase double staining.

Materials and Methods

Patients

Liver biopsy specimens were obtained from 35 patients with persistent HBs antigenemia. The histological diagnoses were non-specific reaction (NSR) in 5 cases, non-specific reactive hepatitis (NSRH) in 5, chronic inactive hepatitis (CIH) in 5, chronic active hepatitis (CAH) in 14 and liver cirrhosis (LC) in 6. HBeAg was positive in 21 cases and anti-HBe was positive in the remaining 14, by radioimmunoassay.

Antisera

Polyclonal rabbit anti-HBs and anti-HBc were commercially obtained (DAKO, Copenhagen, Denmark). The monoclonal antibodies against HLA antigens and lymphocyte subsets used in the present study were as follows: anti-HLA-A,B,C, anti-HLA-DR (Immunotec), anti-CD5 (anti-Leu1), anti-CD8 (anti-Leu2a), anti-CD4 (anti-Leu3a), and anti-Leu7 (Becton-Dickinson FACS System, Sunnyvale, California, U.S.A.).

Goat-anti-mouse immunoglobulins and peroxidase conjugated mouse IgG (Ortho Pharmaceutical Corp, Raritan, New Jersey, U.S.A.) and peroxidase conjugated goat anti-rabbit immunoglobulin G (DAKO) were also obtained commercially.

Light and electron microscopic tissue preparation

Liver biopsy specimens were fixed with periodiate-lysine-2% paraformaldehyde solution for 4 hours, according to the method of McLean and Nakane[13]. After fixation, the specimen was embedded in OCT compound (Miles, Elkhart, USA), immediately frozen in isopentane cooled by liquid nitrogen, and stored at −80°C.

Serial sections of 6μm in thickness were cut with a cryostat and treated with 0.005M periodic acid and 0.1mg/ml of sodium borohydrate to inactivate the endogenous peroxidase activity.

For staining of HBsAg and HBcAg, the indirect immunoperoxidase method was used. Anti-HBs and anti-HBc antibodies were applied at a working dilution of 1:400 for 30 minutes at room temperature. After washing, peroxidase-conjugated goat anti-rabbit IgG was applied for 30 minutes at room temperature. The sections were then stained with 20mg/100ml of diaminobenzidine (DAB) and hydrogen peroxide (H_2O_2)(0.01 ml of 5%H_2O_2: 100ml DAB) solution, dehydrated and mounted on slides for light microscopy.

For staining of HLA antigens and lymphocyte subsets, the sections were incubated with anti-HLA-A,B,C, anti-HLA-DR, anti-CD5, anti-CD8, anti-CD4, and anti-Leu7 antibodies for 12 hours at 4°C, at a dilution of 1:20, 1:80, 1:50, 1:50, 1:80, respectively. After washing, the sections were further applied with goat-anti-mouse immunoglobulins for 2 hours at room temperature, followed by staining with peroxidase conjugated mouse IgG.

Immunoperoxidase double staining was performed for simultaneous demonstration of HBV antigens and HLA antigens, or HBV antigens and lymphocyte subsets on the same sections. DAB (brown) was the first substrate and 4-Cl-1-Naphthol (blue) was the second substrate. After the first staining with DAB, antiserum was removed by 0.1M glycine-HCl at pH2.2[14].

For immunoelectron microscopy, the sections were further fixed with 2.5% glutaraldehyde and 2% OsO4 solutions, respectively, dehydrated in graded ethanols and embedded in epoxy-resin in gelatin capsules. Ultrathin sections were cut on an LKB-8800 ultramicrotome and observed with a Hitachi H-300 electron microscope.

Assessment of degree and localization of HBV and HLA antigens expression on hepatocytes, and of lymphocyte subsets

To evaluate viral expression, the amount of

Table 1 Summary of histological and immunohistochemical findings

Hist. Dx.	Case No.	HBeAg/ anti-HBe	serum GPT	HBsAg		HBcAg		HLA Ag(+) hepatocyte (%)		Number of Lymphocytes (/mm²)	
				score	local.	score	local.	A, B, C	DR	CD8(+)	CD4(+)
NSR	1	+/−	8	4+	M+c	3+	N	< 1.0	0	27	11
(N=5)	2	+/−	10	4+	M+c	2+	N	< 1.0	0	24	15
	3	+/−	20	4+	M+c	2+	N+c	< 1.0	0	30	18
	4	−/+	17	3+	m+C	−		< 1.0	0	28	20
	5	−/+	20	4+	m+C	−		< 1.0	0	62	13
NSRH	6	+/−	12	4+	M+c	3+	N+c	1.5	0	40	19
(N=5)	7	+/−	16	4+	M+c	3+	N+c	< 1.0	0	53	17
	8	+/−	53	4+	M+c	2+	n+c+m	13.8	0	57	29
	9	+/−	55	4+	M+c	3+	N+c	4.6	0	54	23
	10	+/−	66	4+	M+c	2+	n+c+m	9.3	0	70	26
CIH	11	+/−	32	4+	M+c	3+	n+c+m	7.4	0	58	15
(N=5)	12	+/−	40	4+	M+c	1+	c	4.8	<1.0	85	26
	13	+/−	55	4+	M+c	2+	n+c	10.3	0	100	20
	14	−/+	26	2+	m+c	−		2.7	0	48	14
	15	−/+	30	3+	m+c	−		3.0	0	70	18
CAH	16	+/−	74	4+	M+C	2+	n+c+m	19.0	0	76	33
(N=14)	17	+/−	89	2+	m+C	2+	n+c	34.4	<1.0	225	27
	18	+/−	131	3+	M+c	1+	n+c	43.2	<1.0	192	18
	19	+/−	188	4+	M+c	3+	n+c	47.5	<1.0	160	55
	20	+/−	207	4+	M+c	−		19.8	<1.0	212	41
	21	+/−	265	4+	M+c	3+	n+c	37.3	3.2	153	14
	22	+/−	544	3+	m+C	−		23.7	0	203	41
	23	−/+	13	3+	m+C	−		18.2	0	102	30
	24	−/+	63	2+	m+c	1+	c	24.7	<1.0	83	14
	25	−/+	83	4+	M+C	−		31.4	0	203	47
	26	−/+	94	3+	m+c	2+	n+c	20.5	0	72	33
	27	−/+	111	2+	c	1+	c	27.3	0	186	25
	28	−/+	112	4+	M+c	−		55.1	<1.0	154	45
	29	−/+	170	4+	M+c	−		49.0	2.3	175	38
LC	30	+/−	8	3+	M+c	1+	n+c	18.6	<1.0	101	44
(N=6)	31	+/−	19	4+	M+c	2+	n+c	39.3	4.7	69	20
	32	+/−	23	3+	C	1+	n+c	28.1	0	48	52
	33	−/+	24	1+	c	−		25.9	<1.0	60	44
	34	−/+	37	2+	c	−		47.7	3.7	87	25
	35	−/+	89	4+	m+C	1+	n	33.5	5.4	124	22

NSR: non specific reaction, NSRH: non specific reactive hepatitis, CIH: chronic inactive hepatitis, CAH: chronic active hepatitis, LC: hepatic cirrhosis.
score: degree of HBV associated antigen positive hepatocyte (see text). loca.: localization
M,m: membranous, C,c: cytoplasmic. N,n: nuclear. (the capital reflect more than a half of hepatocytes showing the same localization.)

hepatocyte expressing viral antigen in each case was scored on a scale ranging from − to 4+ (−: 0%, 1+: <5%, 2+: <30%, 3+: <80%, 4+: ≧80% of hepatocytes containing HBV antigens in whole liver biopsy specimen of individual cases). The localization of the viral antigen in hepatocytes was classified as membranous (m), cytoplasmic (c) or nuclear (n). When more than half of hepatocytes showed the same pattern of localization, the pattern was represented with

the capital letter of the classification (e. g. M).

For quantitative assessment of the hepatocytes expressing HLA-A,B,C antigens or HLA-DR antigens, the number of antigenpositive hepatocytes in a $2mm^2$ area of the biopsy specimens was counted in each case and the frequency was calculated.

The distribution of lymphocyte subsets were observed and especially, the numbers of CD8(+) and CD4(+) lymphocytes in $1mm^2$ areas of liver specimens (except the portal tract) in each case were counted. (**Table 1**)

In addition, the distribution pattern of HBV antigens, HLA antigens on hepatocytes and of lymphocyte subsets were compared on serial sections and double-stained specimens of each case.

Results

1. Localization of HBsAg

HBsAg was displayed in the cytoplasm in all 35 liver biopsy specimens and in the liver cell membrane in 31 (31/35:89%). In 3 cases of NSR, 5 of NSRH, 3 of CIH, 8 of CAH and 2 of LC HBsAg predominated in the liver cell membrane and most of them (18/21:86%) were HBeAg positive in sera (**Table 1**).

2. Localization of HBcAg

HBcAg was detected in 23 liver specimens (23/35: 66%, 3 NSR; 5 NSRH; 8 CAH; 4 LC). Nineteen cases were HBeAg-positive and 4 were anti-HBe positive in sera. In contrast in cases of NSR, this antigen was observed both in the nucleus and cytoplasm or in the cytoplasm alone in cases of chronic liver disease. Membranous localization of HBcAg at the light microscopic level was found in 2 cases of NSRH and in one case each of CIH and CAH (**Table 1**).

3. Expression of HLA-A, B, C Antigens

HLA-A,B,C antigens on the sinusoidal lining cells, Kupffer cells and endothelial cells, were expressed similarly in all cases (NSR to LC). On the contrary, the expression of HLA-A,B,C antigens on hepatocytes was different between cases. In cases of NSR, HLA-A,B,C antigens were hardly demonstrated on hepatocytes, but on sinusoidal lining cells, showing so-called sinusoidal pattern. In cases of liver disease, HLA-A,B,C antigens were markedly demonstrated on hepatocyte plasma memgbranes, showing a honey-comb pattern. The percentage of HLA-A,B,C antigens positive hepatocytes were less than 1.0% in NSR, 6.0±5.5% in NSRH, 5.6±3.2% in CIH, 32.2±12.4% in CAH and 32.2±10.3% in LC. These perentages were significantly higher in the cases of liver disease compared with cases of NSR ($P<0.01$). Furthermore, the percentages in cases of CAH and LC were significantly higher than those in cases of NSRH and CIH ($P<0.01$). These HLA-A,B,C antigens positive hepatocytes were located in and around the necrotizing inflammatory area such as piecemeal necrosis and focal necrosis (**Figs. 1a and 2b**).

Analysis of all cases showed that the frequency of HLA-A, B, C, antigen-positive hepatocytes(y) correlated positively with the number of infiltrating CD8(+) lymphocytes(x) ($y=0.18x+2.05$, $r=0.665$, $r^2=0.443$, $P<0.01$) and serum GPT levels(z) ($y=0.064z+15.0$, $r=0.395$ $r^2=0.156$, $P<0.05$).

4. Expression of HLA-DR antigens

Some of mononuclear cells, mainly lymphocytes, in areas of necro-inflammatory lesions and the sinusoidal lining cells composed of Kupffer cells and endothelial cells expressed HLA-DR antigens (**Fig. 1b**). Although the number of hepatocyte expressing HLA-DR antigens was very small, HLA-DR antigens were displayed on some of the hepatocyte membrane of CIH (1/5), CAH (8/14) and LC (4/6) (**Table 1**). HLA-DR antigen-positive hepatocytes were distributed focally and restricted in the necrotizing inflammatory area, and not distributed diffusely like the HLA-A,B,C antigens showing honey-comb patterns. A close association between the HLA-DR antigen-positive hepatocytes and HLA-DR antigen-positive inflammatory cells was also found (**Fig. 1b**).

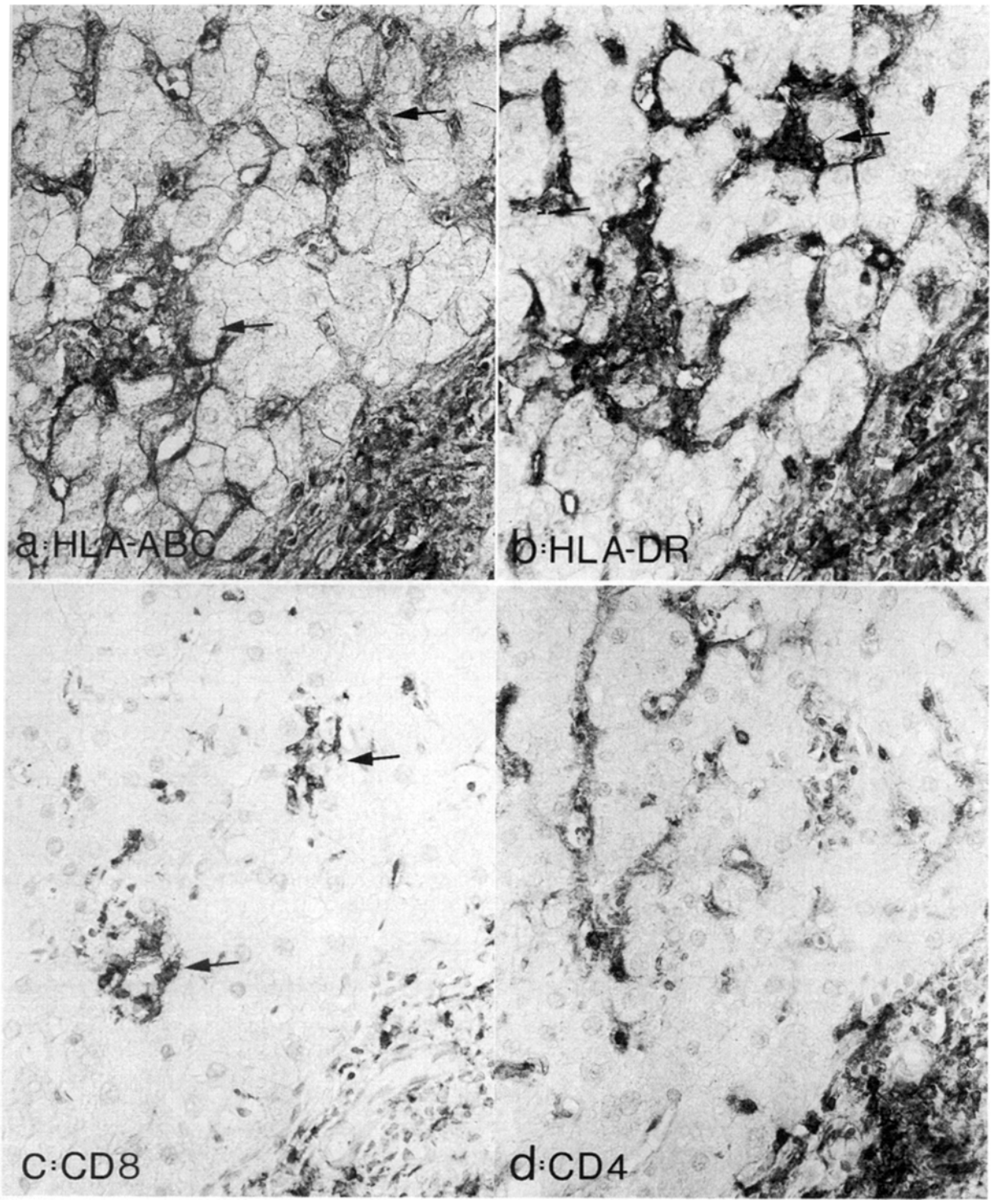

Fig. 1 Focal necrosis adjacent to the portal tract in a case of CAH. From 'a' to 'd' are serial sections. (a): HLA-A,B,C antigens are positive on the plasma membrane of hepatocytes, showing honey-comb pattern in and around the areas of focal necrosis (arrows). (b): HLA-DR antigens are positive on the plasma membrane and the cytoplasm of only a few hepatocytes in the areas of focal necrosis (arrow). (c): CD8(+) lymphocytes accumulated in the areas of focal necrosis (arrows). (d): CD4(+) lymphocytes are rare in the lobule, but are abundant in the portal tract. Immunoperoxidase staining with weak hematoxylin staining. (×350)

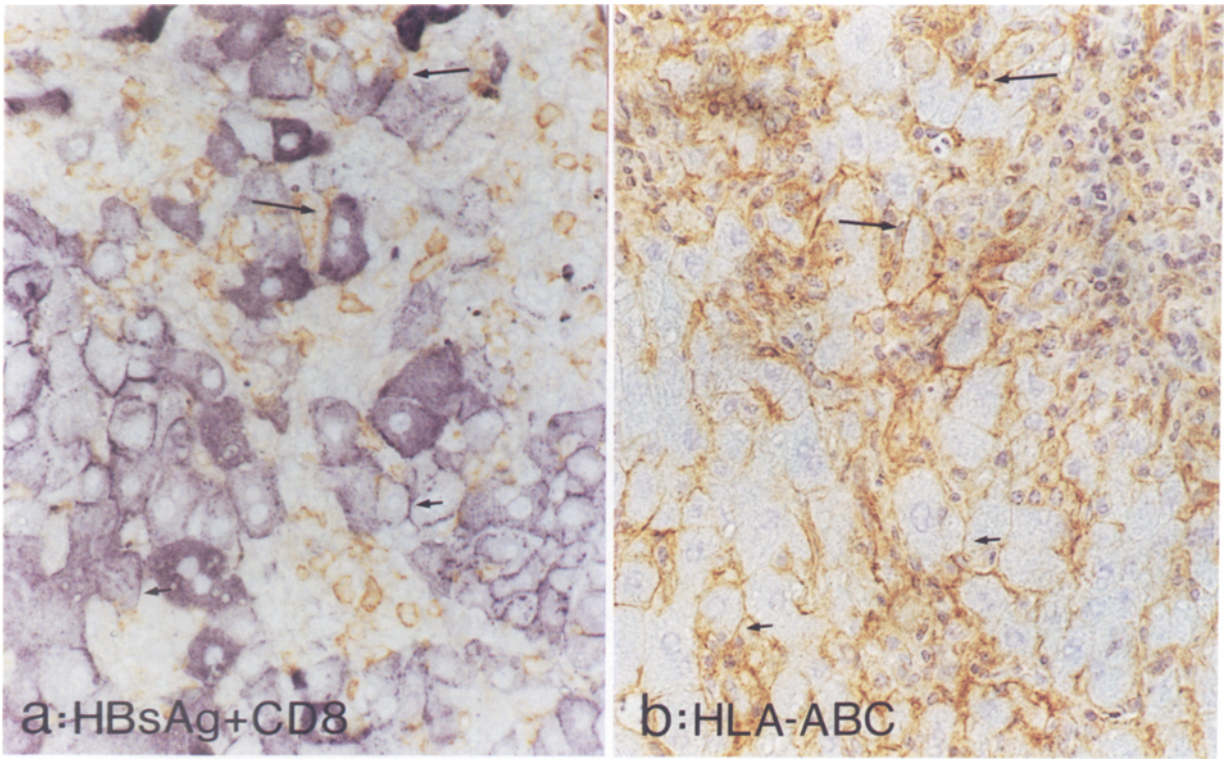

Fig. 2 The areas of piecemeal necrosis of CAH with serum HBeAg positive. 'a' and 'b' are strict serial sections (arrows indicate the same cells). (a): Diffuse membranous and sporadic cytoplasmic localization of HBsAg (blue) and abundant CD8(+) lymphocytes (brown). (b): HLA-A,B,C antigens on the plasma membrane of hepatocyte in and around the areas of piecemeal necrosis. Both of HBsAg and HLA-A,B,C antigens are positive on the plasma membrane of the same hepatocytes ('a' and 'b': short arrows) and some of these hepatocytes show contact with lymphocytes ('a' and 'b': long arrows). b: immunoperoxidase staining for HLA-A,B,C, and hematoxylin staining. (×350)

5. Distribution of lymphocyte subsets

Abundant CD5(+) lymphocytes were demonstrated in specimens of cases of liver diseases. CD8(+) lymphocytes were demonstrated mainly in the lobule and periportal area, namely in the areas of hepatocyte injury. On the contrary, CD4(+) lymphocytes were demonstrated mainly in the central part of the portal area (**Fig. 1d**). The number of CD8(+) lymphocytes in 1mm^2 specimens apart from the portal tract were 34.2±15.7 in NSR, 54.8±10.7 in NSRH, 72.2±30.8 in CIH, 156.9±53.1 in CAH and 81.5±28.1 in LC, respectively. The number of CD4(+) lymphocytes were 15.4±3.6 in NSR, 22.8±4.9 in NSRH, 18.6±4.8 in CIH, 32.9±12.5 in CAH and 34.5±13.7 in LC, respectively. The differences between the mean number of CD4(+) lymphocytes of each groups were smaller than those for CD8(+) lymphocytes. However, a significant increase was demonstrated in CAH and LC compared with NSR ($P<0.01$). In addition, Leu7(+) lymphocytes were less than 5 per mm^2 in all sections of liver tissue in all groups.

6. Coexpression of HBV antigens and HLA antigens

Simultaneous localization of HBV antigens and HLA antigens was performed by an immunoperoxidase double staining method using the two different substrates of diaminobenzidine (brown) and 4-C1-1-Naphthol (blue). In NSR, HLA-A,B,C antigens were observed in a small number of hepatocytes in spite of the diffusely located HBsAg and HBcAg (**Fig. 4a**). Furthermore, HLA-DR antigens were completely negative. In cases of chronic liver dis-

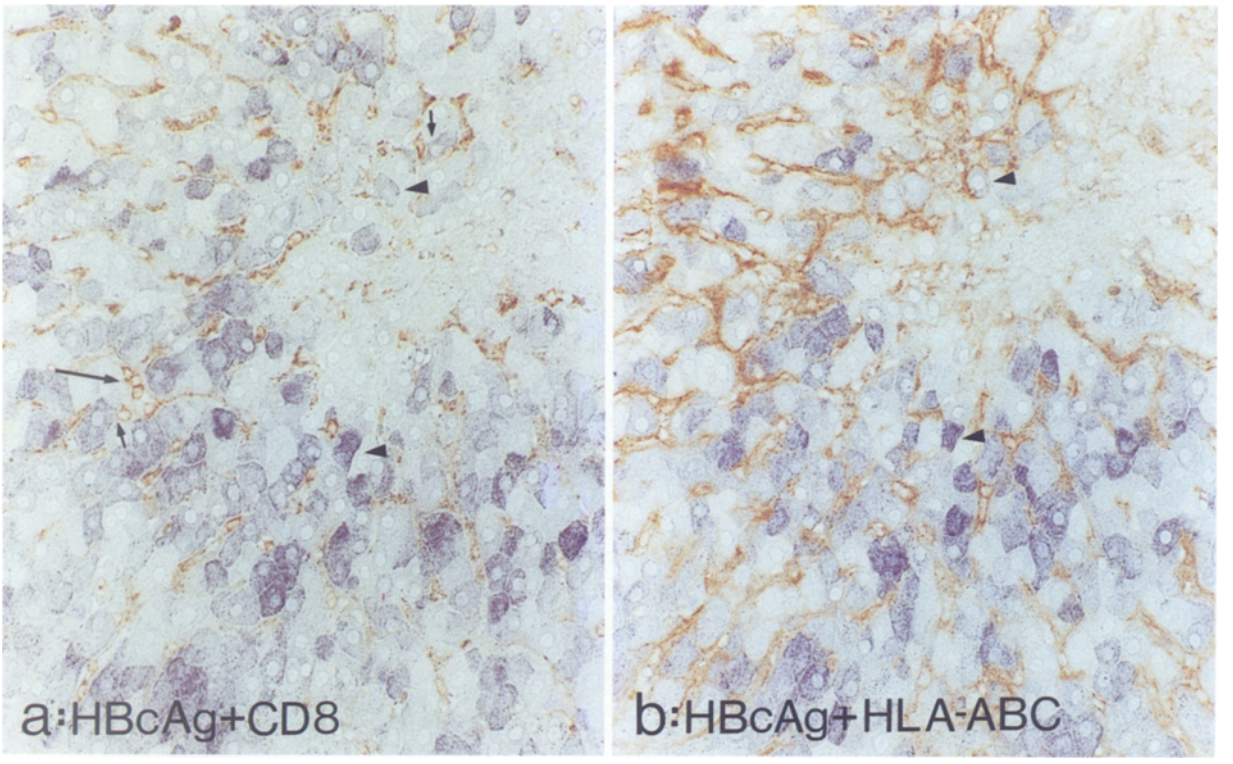

Fig. 3 Periportal area of CAH with serum HBeAg-positive. 'a' and 'b' are strict serial sections (arrow heads indicate the same cells). (a): Cytoplasmic localization of HBeAg (blue) and CD8(+) lymphocytes (brown). (b): Cytoplasmic localization of HBcAg (blue) and membranous localization of HLA-A,B,C antigens (brown). HBcAg positive hepatocytes are distributed at random in the lobule. Some of them express HLA-A,B,C antigen on the plasma membrane. CD8(+) lymphocyte shows contact with HBcAg-positive hepatocyte (short arrow) and -negative hepatocyte (long arrow). Immunoperoxidase double staining. (×200).

ease with HBeAg-positive in serum, HBV antigens and HLA-A,B,C antigens were coexpressed on the hepatocytes in areas of piecemeal necrosis and focal necrosis. Particularly, HBsAg and HLA-A,B,C antigens were coexpressed on the plasma membrane of the same hepatocytes in areas of necrosis (**Fig. 2a, b**). Hepatocytes expressing cytoplasmic HBcAg and HLA-A,B,C, antigens were also found in these necro-inflammatory areas, though HBcAg were distributed nearly at random (**Fig. 3**). In addition, some lesions showed hepatocytes expressing only HLA-A,B,C antigens and other lesions showed hepatocytes expressing cytoplasmic HBcAg and HLA-A,B,C antigens (**Fig. 4b**).

Although most cases of anti-HBe positive in serum were free of HBcAg in biopsy specimens, HLA-A,B,C, antigens were similarly displayed in cases of chronic liver disease (**Table**). Hence there was no direct relation with HBV infection in term of the expression of HLA-A,B,C antigens.

The numbers of hepatocytes co-expressing HBV antigens and HLA-DR antigens were small, because of the small number of hepatocyte expressing HLA-DR antigen per se. The hepatocytes expressing HLA-DRA antigens showed various types of localization of HBV antigens (**Fig. 5**).

7. Immunoelectron microscopic finding of HLA antigens expression

HLA-A,B,C antigens were demonstrated only on the plasma membrane and some ER of sinusoidal lining cells in cases of NSR (**Fig. 6**). In cases of liver disease, HLA-A,B,C antigens were also demonstrated on hepatocytes, whole

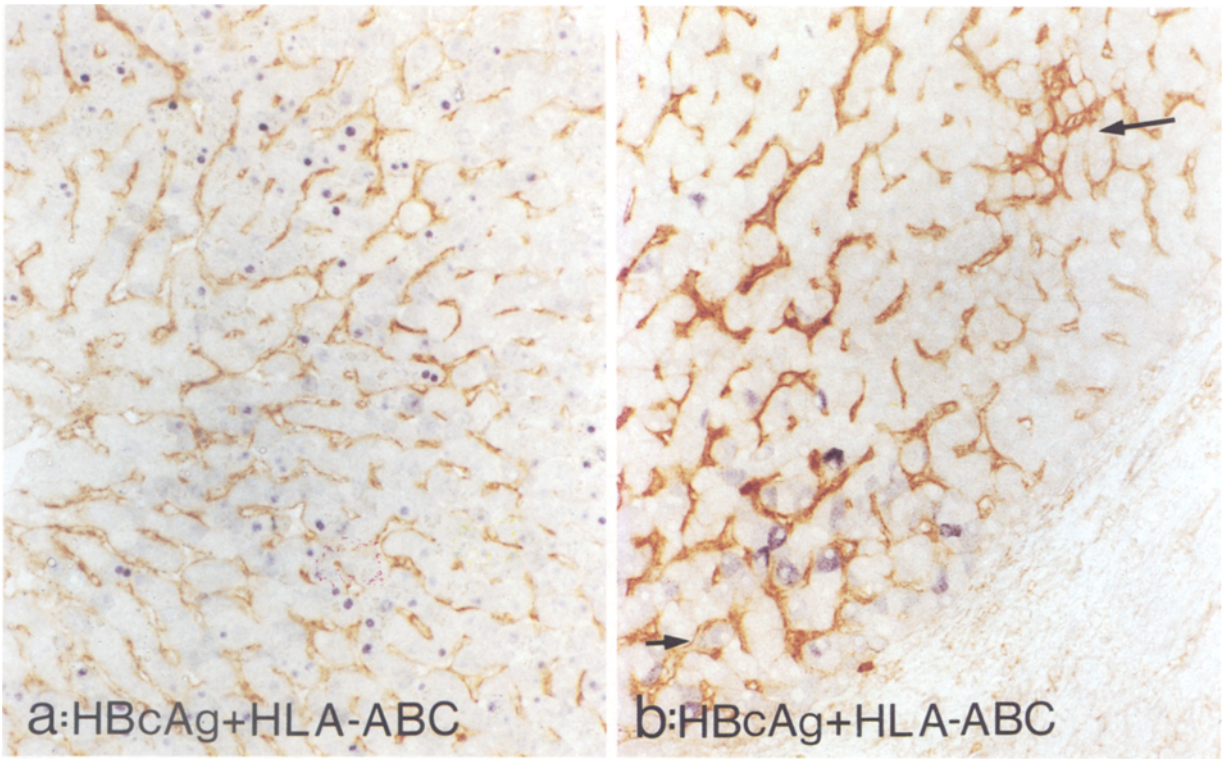

Fig. 4 Comparison of the localization of HBcAg (blue) and HLA-A,B,C antigen (brown). (a): Diffuse nuclear localization of HBcAg and no HLA-A,B,C positive hepatocyte (so-called sinusoidal pattern) in a case of NSR with serum HBeAg-positive. (b): one region (short arrow) shows HLA-A,B,C-positive hepatocytes containing cytoplasmic HBcAg and another region shows HLA-A, B, C-positive heaptocytes, showing honey comb pattern (long arrow) without HBcAg in a case of hepatic cirrhosis with serum HBeAg-positive. Immunoperoxidase double staining. (×200)

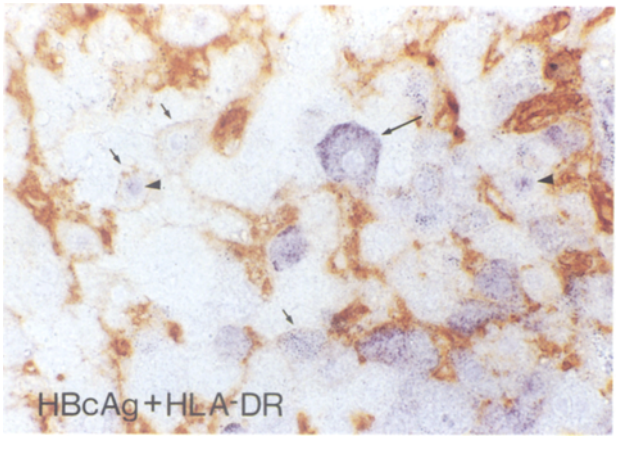

Fig. 5 Comparison of the localization of HBcAg (blue) and HLA-DR (brown) in a case of HBeAg-positive CAH. Cytoplasmic (long arrow) and nuclear (arrow heads) localization of HBcAg. Some HLA-DR positive hepatocytes contain HBcAg (short arrows). Immunoperoxidase double staining. (×250)

hepatocyte plasma membrane except for the biliary canalicular surface, and in some ER near plasma membrane (**Fig. 7**).

The expression of HLA-DR antigens on hepatocytes was rare, but was confirmed by immunoelectron microscopy. HLA-DR antigens were observed to be relatively abundant in the ER of hepatocytes (**Fig. 8**) in contraast to less frequent localization of HLA-A,B,C antigens.

8. Expression of HLA antigens and lymphocyte interaction

Most of the lymphocytes in the areas of piecemeal necrosis and focal necrosis were CD8(+) cells. At the light microscopic level, hepatocytes expressing HBsAg and HLA-A,B,C antigen on the plasma membrane showed contact with CD8(+) cells in contact with hepatocytes and hepatocytes expressing HLA-A, B, C antigens

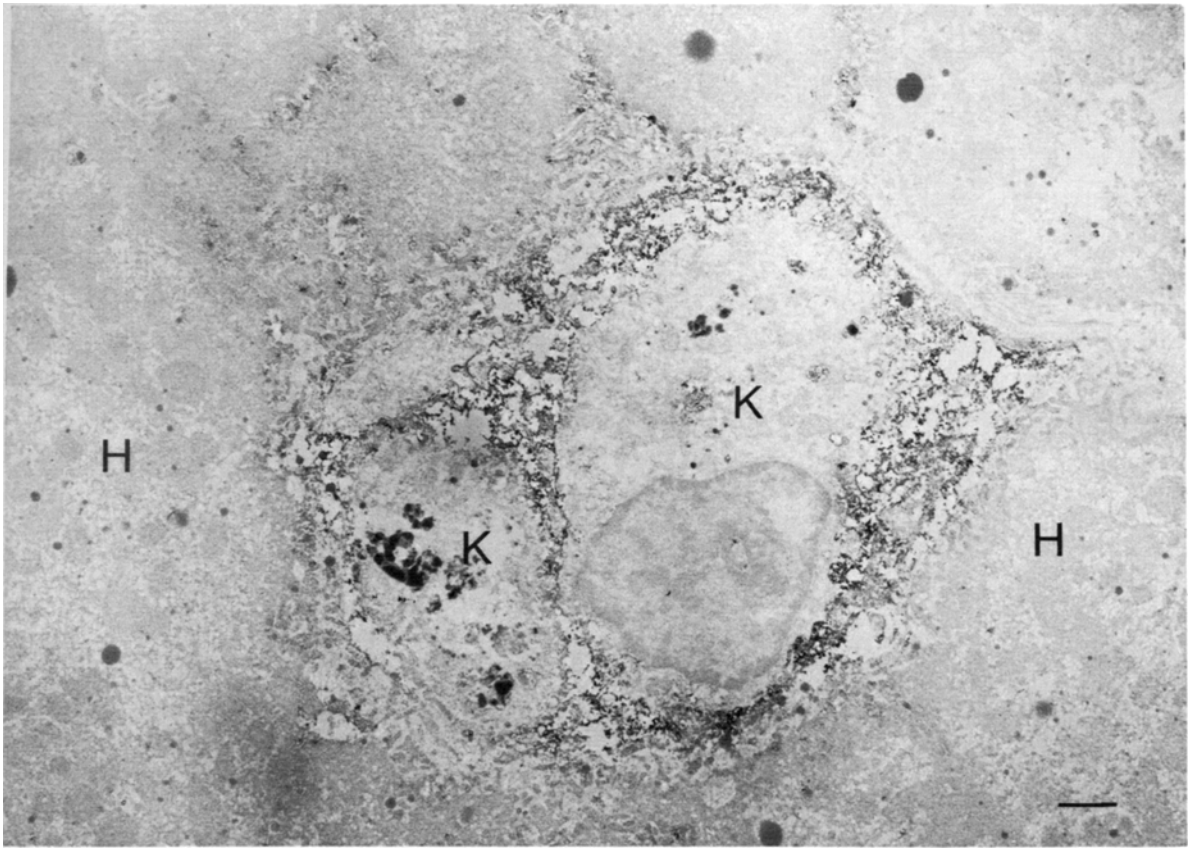

Fig. 6 Immunoelectron micrograph of HLA-A,B,C antigens in a case of NSR. HLA antigens are demonstrated on the plasma membrane and ER of Kupffer cells (K), but not on hepatocytes (H). (×7300)

in contact with lymphocytes also positive for these antigens were demonstrated (**Fig. 9**).

Discussion

HLA molecues have been noted to be transmembrane glycoproteins, important in cell-to-cell communication as receptor[15]. In 'in vivo' experiments, viral infection altered (enhanced or suppressed) the expression of HLA antigens on a variety of culture cells[10,11]. In this sense, hepatitis B virus (HBV) is also considered to play a causative role in HLA antigen expression on infected hepatocytes. On the other hand, recently accumulated date suggest the immune mechanism of T-cell mediated cell lysis in type B hepatitis. In generating the T-cell mediated cytotoxicity, the membrane expression of HLA-A,B,C, antigens along with viral antigens is required[9].

Immunofluorescent and immunoperoxidase studies demonstrated HLA-A,B,C antigens on lymphocytes of peripheral blood, lymphoid cells of lymphatic organs, endothelial cells of small vessels and transitional epithelial cells of glomeruli, etc[16,17].

In normal liver, HLA-A,B,C antigens are not demonstrated on hepatocyte plasma membrane or cytoplasm, but are present on the cell membrane of bile duct epithelium, sinusoidal lining cells, fibroblasts and blood vessel endothelium. In case of various liver diseases, HLA-A, B, C antigens were demonstrated on the hepatocyte plasma membrane[18].

Montano et al[12] were the first to analy the expression of HLA antigens in livers of patients with chronic HBV infection. They studied the expression of HLA antigens both in serum anti-

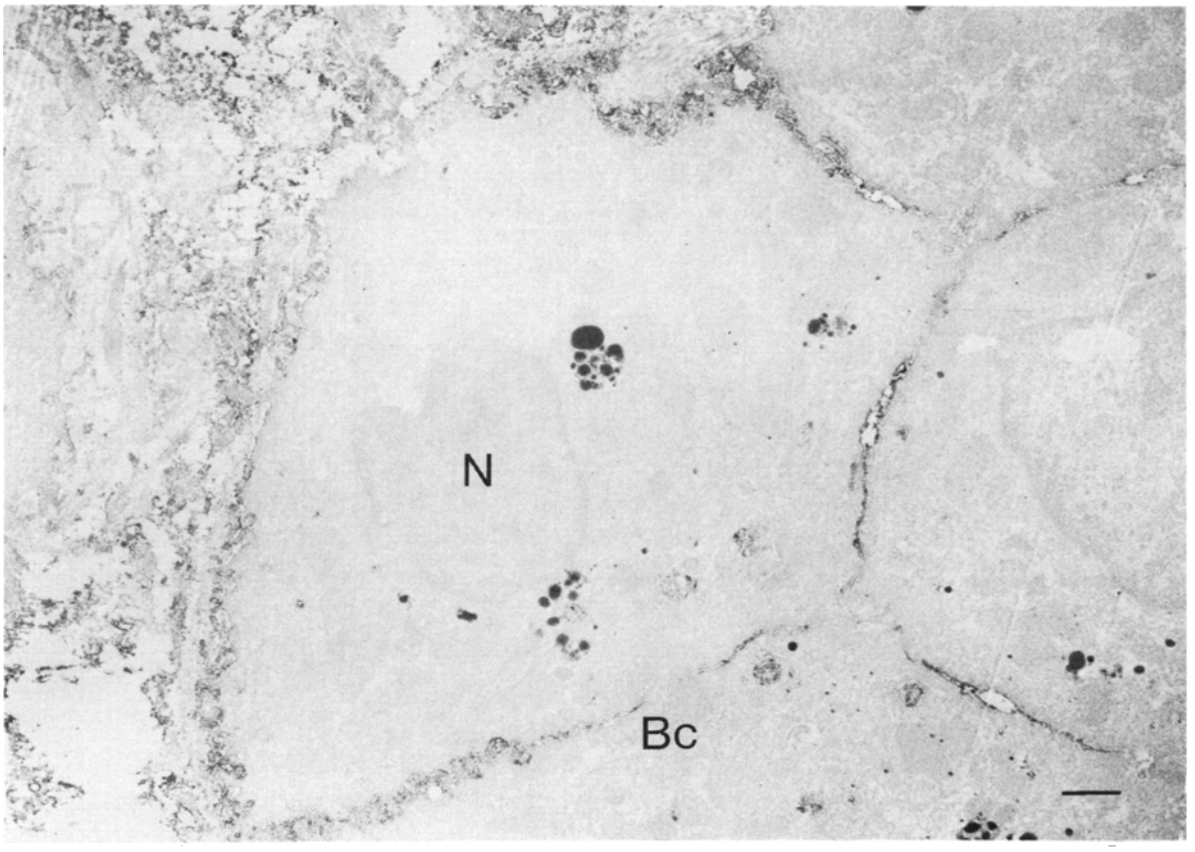

Fig. 7 Immunoelectron micrograph of HLA-A,B,C antigens on hepatocytes. HLA-A,B,C antigens are demonstrated at the sinusoidal and lateral membranes and some of ER (arrows). No reaction products on the bile canalicular membrane are found. N: nucleus, Bc: bile canaliculus. (×7700)

HBe-positive cases and HBeAg-positive cases by the immunofluorescence method. Increased expression of HLA-A, B, C antigens on hepatocytes in cases with serum anti-HBe positive was found in comparison with decreased expression in serum HBeAg-positive cases. These discrepancies in the expressions of HLA antigens and viral antigens were concluded to be an important factor in determining the chronicity of HBV infection. Recently, Fukusato et al.[19] reported a significant correlation between the expression of HLA-A,B,C antigens on hepatocytes and the intensity of intralobular inflammation in a study of various liver diseases. However, only a small number of hepatocytes expressing both HLA-A,B,C antigens and HBV antigens (HBsAg, HBsAg) were observed by using a double immunofluorescent method and they concluded no specific relation between HLA-A, B, C antigens and HBV antigen expression.

In the present study, HBsAg was diffusely distributed on the majority of hepatocytes including necrotizing inflammatory areas, whereas HLA-A,B,C antigens were found relatively restricted in areas of piecemeal necrosis and focal necrosis. On the other hand, intracytoplasmic HBcAg was observed focally in necrotizing inflammatory areas; HBcAg and HLA-A, B, C antigens were frequently co-localized in the same hepatocytes of these lesions. Concerning the HLA-A,B,C antigens, the intensity of antigen expression on hepatocytes did not differ from cases with werum HBeAg positive and those with serum anti-HBe positive. This suggests no particular relation be-

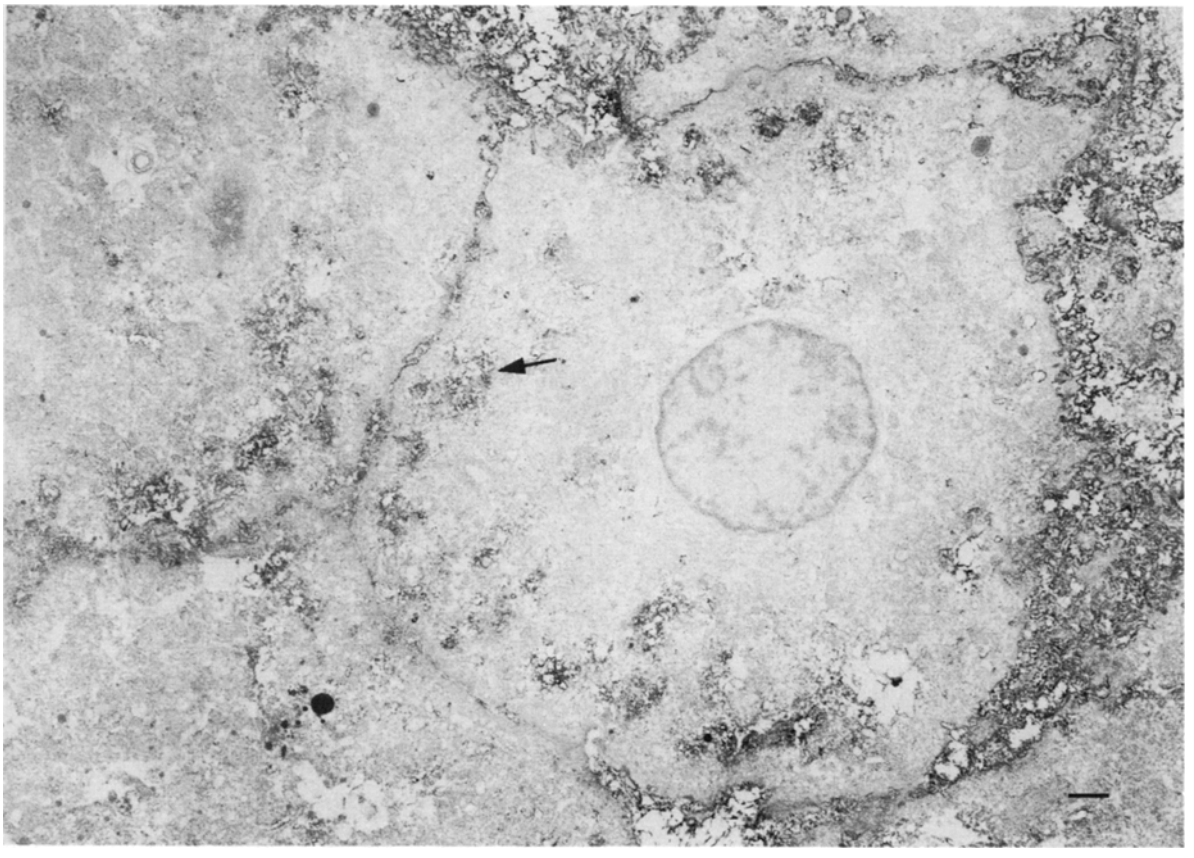

Fig. 8 Immunoelectron micrograph of HLA-DR antigens on hepatocytes. HLA-DR antigens are demonstrated on the plasma membrane and some of ER (arrow). (×4700)

tween HLA-A,B,C antigens expression and HBV infection. On the contrary, the percentages of hepatocytes expressing HLA-A, B, C corellated well with the infiltration of CD8(+) lymphocytes in the liver. Therefore, it was suggested that HLA-A,B,C antigen expression on hepatocytes was not influenced by HBV infection itself, but was related to hepatocyte lysis by CD8(+) lymphocytes.

The increased number of hepatocytes expressing both HLA-A,B,C antigens and viral antigens may allow cytotoxic T-lymphocyte to recognize and eliminate these hepatocytes in the serum HBeAg-positive phase of hepatitis B. Especially, CD8(+) lymphocytes showed direct contact with the hepatocytes expressing both HBsAg and HLA-A,B,C antigens on the plasma membrane. At the immunoelectron microscopic level direct contact of CD8(+) lymphocytes with such hepatocytes was confirmed[8]. These findings fulfilled the morphological evidence of HBsAg as a target.

On the other hand, several investigators have supposed HBcAg to be the target antigen. Studies based on the autologous lymphocyte toxicity test suggest that HBcAg is the target antigen for T-cell mediated cytotoxicity, because of a significant inhibition of the cytotoxic reaction after adding anti-HBc[4]. Furthermore, HBcAg on the liver cell plasma membrane of isolated hepatocytes was detected after antibody elution with high molar urea[5]. By immunoelectron microscopy, Kojima et al.[20] demonstrated HBcAg on the plasma membrane of hepatocytes in chronic HBV infection. In the present study, HBcAg was demonstrated on the

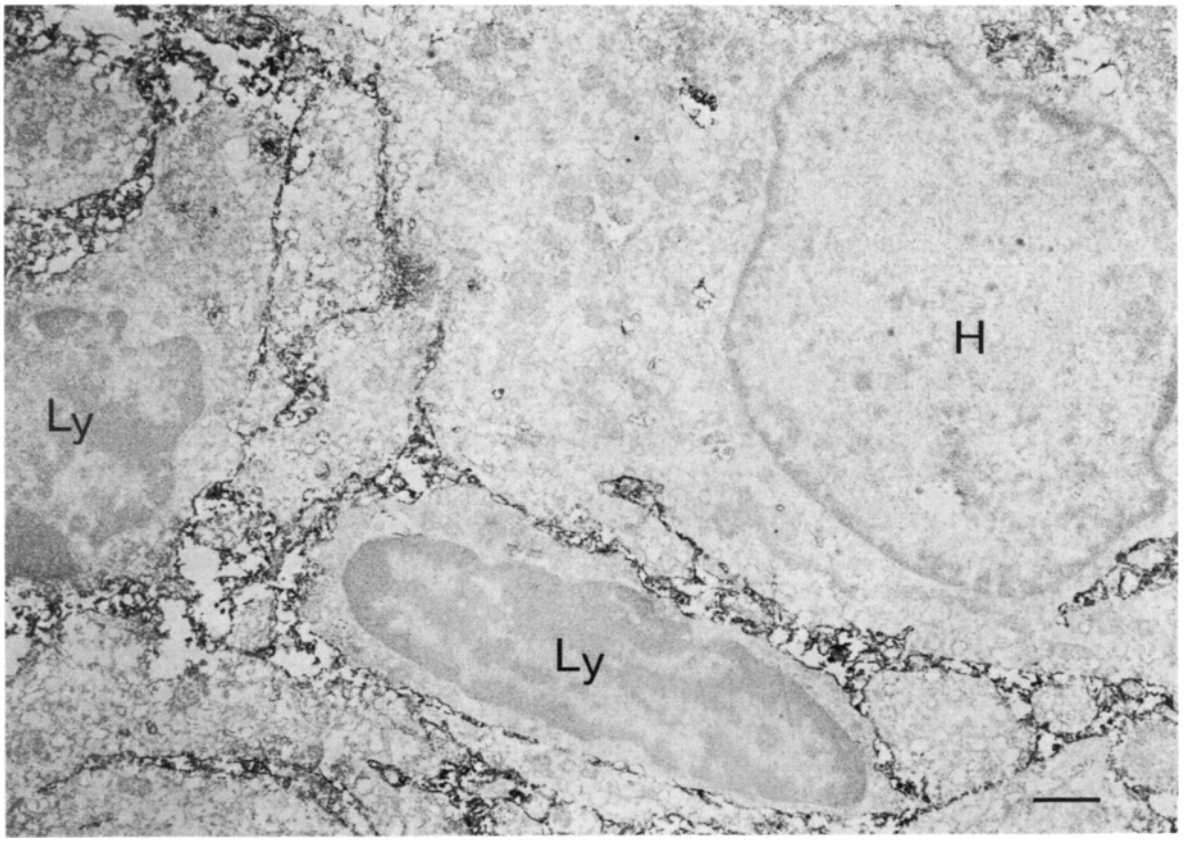

Fig. 9 Immunoelectorn micrograph of HLA-A,B,C antigens on plasma membrane of both hepatocyte (H) and lymphocytes (L). Extensive contact between HLA-A,B,C antigen positive hepatocyte and lymphocytes. (×9000)

plasma membrane in the minority of hepatocytes in a few cases at the light microscopic level, therefore it may not be reasonable to consider that HBcAg is the main target viral antigen. The localization of HBcAg tended to be in the cytoplasm in HBeAg-positive of CAH cases, compared with nuclear localization in cases of NSR with HBeAg. Contact of CD8(+) lymphocytes with hepatocytes containing cytoplasmic HBcAg may reflect membranous expression of new antigen induced by HBV infection. Further investigation is needed to elucidate this possibility.

In contrast to HLA-A, B, C antigens, HLA-DR antigens have been considered not to be expressed on the hepatocyte membrane in normal or diseased liver[12,19]. However, recently, van den Oord et al.[21] successfully demonstrated HLA-DR antigens on the liver cell membrane in cases of CAH, and emphasized the preferential association between HLA-DR antigens on the liver cell membrane and HBcAg in the nucleus of the same hepatocytes in areas of spotty necrosis, but not in areas of piecemeal necrosis. They claimed that γ-interferon secreted by lymphocytes in areas of spotty necrosis might induce the HLA-DR antigens. In agreement with the van den Oord findings, in the present study hepatocytes expressing both HLA-DR antigens and HBV antigens were demonstrated in areas of spotty necrosis. Unlike the van den Oord study, however, HLA-DR antigens were demonstrated not only in areas of focal necrosis but also in areas of piecemeal necrosis in the present study. In addition, no significant corellation between the distribution of HLA-DR antigens and viral antigens was found. HLA-DR antigens on hepatocytes may allow HLA-DR-

restricted CD4(+) T cells to recognize viral antigens on the hepatocytes. However, hepatocyte lysis mediated by CD4(+) T cells can be thought to be a minor immune mechanism in hepatitis B because of the small number of hepatocytes expressing HLA-DR antigens and also the minor component of CD4(+) cells in necrotizing inflammatory lesions.

It was been clarified that several lymphokines could induce the expression of HLA antigens on several kinds of cells[22]. Franco et al.[23] using primary cultured human hepatocytes demonstrated that α-, and γ-interferons induced the expression of HLA-A;B,C antigens, and γ-interferon also induced the expression of HLA-DR antigens. When the surface markers were examined, it was shown that the major population of γ-interferon-producing cells were CD3(+), CD4(+), CD8(−) cells (helper/inducer T cells)[24]. Although CD4(+) cells are minor components in areas of necro-inflammatory lesions in liver biopsies of hepatitis B, CD8(+) cytotoxic T cell-mediated cytotoxicity may need the help of CD4(+) cells in regard to the induction of expression of HLA antigens on hepatocytes.

References

1. Dudley FJ, Fox RA, Sherlock S: Cellular immunity and hepatitis-associated australia antigen liver disease. Lancet 1972;1: 723-726
2. Ray MB, Desmet VJ, Bradburne AF, et al: Differential distribution of bepatitis B surface antigen and hepatitis B core antigen in liver of hepatitis B patients. Gastroenterology 1976;71: 462-467
3. Alberti A, Realdi G, Bortolitti F, et al: T-lymphocyte cytotoxicity to HBsAg-coated target cells in hepatitis B virus infection. Gut 1977;18:1004-1009
4. Eddleston ALWF, Mondelli M, Miele-Vergini G, et al: Lymphocyte cytotoxicity to autologous hepatocytes in chronic hepatitis B virus infeciton. Hepatology 1982;2:122S-127S
5. Trevisan A, Realdi G, Alberti A, et al: Core antigen-specific immunoglobulin-G bound to the liver cell membrane in chronic hepatitis B. Gastroenterology 1982;82:218-222
6. Eggink HF, Houthoff HJ, Heutema S, et al: Cellular and humoral immune responses in chronic active liver disease. I. Lymphocyte subsets in liver biopsies of patients with untreated idiopathic autoimmune hepatitis, chronic active hepatitis B and primary biliary cirrhosis. Clin Exp Immunol 1982;50:17-24
7. Montano L, Aranguibel F, Boffill M, et al: An analysis of the composition of the inflammatory infiltrate in autoimmune and hepatitis B virus-induced chronic liver disease. Hepatology 1983;3: 292-296
8. Sasaki H, Kojima T, Matsui S, et al: Interaction of lymphocytes with hepatocytes containing hepatitis B antigen: ultrastructural demonstration of target antigen and T-cell subsets by the peroidase antibody technique. Virchow Arch A 1987;411:489-498
9. Meuer SC, Schlossman SF, Reinherz EL, et al: Clonal analysis of human cytotoxic T lymphocytes: T4(+) and T8(+) effector T cells recognize products of different major histocompatibility complex regions. Proc Natl Acad Sci 1982;79:4395-4399
10. Schrier PI, Bernard R, Veresen RTMJ, et al: Expression of class I major histocompatibility antigen switch off be highly oncogenic adenovirus 12 in transformed rat cells. Nature 1983;305: 771-775
11. Flyer DC, Burakoff SF, Faller DV: Retrovirus-induced changes in major histocompatibility complex antigen expression influence susceptibility to lysis by cytotoxic T lymphocytes. J Immunol 1985;135:2287-2292
12. Montano L, Miescher GC, Goodall AH, et al: Hepatitis B virus and HLA antigen display in the liver during chronic hepatitis B virus infection. Hepatology 1982;2:557-561
13. McLean IW, Nakane PK: Periodate-lysine-paraformaldehyde fixative: a new fixative for immunoelectron microscopy. N Histochem Cytochem 1974;22:1077-1083
14. Nakane PK: Simultaneous localization of multiple tissue antigens using the peroxidase-labeled antibody method: a study of pituitary glands of rat. J Histochem Cytochem 1968;16:557-560
15. Benacerraf B, mcDevitt HO: The histocompatibility-linked immune response gene. Sinence 1972;175:273-293
16. Brown G, Biberfeld P, Christensson B, et al: The distribution of HLA on human lymphoid, bone marrow and peripheral blood cells. Eur J Immunol 1979;9:272-275
17. Fleming KA, McMichael A, Morton JA, et al: Distribution of HLA class 1 antigens in normal human tissue and in mammary cancer. J Clin Phthol 1981;34:779-784
18. Barbatis C, Woods J, Morton JA, et al: Immunohistochemical analysis of HLA (A,B,C) antigens in liver disease using a monoclonal antibody. Gut 1981;22:985-991
19. Fukusato T, Gerber MA, Thung SN, et al: Expression of HLA class I antigens on hepatocytes in liver disease. Am J Pathol 1986;123:264-270
20. Kojima T, Bloemen J, Desmet VJ, Immune electron microscopic demonstration of hepatitis B core antigen (HBcAg) in liver cell plasma membranes. Liver 1987;7:191-200
21. Van Denn Oord JJ, De Vos R, Desmet VJ: In site distribution of major histocompatibility complex products and viral antigen in chronic hepatitis B virus infection: Evidence that HBc-containing hepatocytes may express HLA-DR antigens. Hepatology 1986;6:981-986
22. Basham TY, Bourgerde MF, Creasy AA, et al: Interferon increases HLA synthesis in melanoma cells: interferon-resistant and sensitive cell lines. Proc Natl Acad Sci 1982;79:3265-3269
23. France A, Barnaba V, Natali P, et al: Expression of class I and class II major histocompatibility complex antigens on human hepatocytes. Hepatology 1988;3:449-454
24. Martinez-Maza O, Anderson U, Anderson J, et al: Spontaneous production of interferon-γ in adult and newborn humans. J Immunol 1984;132:251-255