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Cellular and humoral immune reactions in chronic active liver disease. II. Lymphocyte subsets and viral antigens in liver biopsies of patients with acute and chronic hepatitis B

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SUMMARY

The characteristics and distribution of the inflammatory infiltrate in liver biopsies of 25 patients with hepatitis B viral (HBV) infection were studied in relation to the distribution and expression of HBV antigens. Mononuclear subsets were characterized with monoclonal (OKT, OKM, Leu) antibodies to surface antigens. For the demonstration of viral antigens directly conjugated antibodies to surface (HBsAg), core (HBcAg) and 'e' (HBeAg) antigen were used. For the study of mutual relations all methods were performed on serial cut tissue sections. In chronic active hepatitis B (CAH-B, $n=12$) OKT8$^+$ lymphocytes of T cell origin were the only cell type present in areas with liver cell degeneration and T cell cytotoxicity appears to be the only immune mechanism. In chronic persistent hepatitis B (CPH-B, $n=7$) the only conspicuous feature was the presence of many Leu 3$^+$ lymphocytes of the helper/inducer population in the portal tracts. In acute hepatitis B (AHB, $n=6$) OKT8$^+$ cells of non-T origin (OKT1$^-$, 3$^-$) and Leu 7$^+$ cells of presumed natural killer (NK) potential predominated in the areas with liver cell necrosis, and non-T cell cytotoxicity appears to be the predominant immune mechanism. In none of these disease entities a positive spatial relation could be established between the cytotoxic cells and the demonstrable expression of HBV antigens in hepatocytes. It is concluded that differences in immunological reaction pattern may explain the different course in the three forms of HBV infection studied.

Keywords chronic active liver disease hepatitis B lymphocyte subsets viral antigens

INTRODUCTION

The contribution of host defence mechanisms and viral antigenic expression in acute and chronic hepatitis B virus (HBV) infection of the liver are still incompletely understood.

As shown by the course of HBV infection during immunosuppression, the virus itself is not cytotoxic for hepatocytes and liver cell damage is related to the immunological reactions patterns of the host (Edgington & Chisari, 1975; Gudat *et al.*, 1975). The expression of HBV surface (HBsAg) or core (HBcAg) antigens on the cell surface or of other HBV-induced alterations of the liver cell membrane have been postulated as the main target antigens for host defence mechanisms

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(Edgington & Chisari, 1975; Ray *et al.*, 1976; Trevisan *et al.*, 1982). Based on *in vitro* studies, cell-mediated cytotoxicity (CMC) or antibody-dependent cell-mediated cytotoxicity (ADCC) are supposed to be the main immune mechanisms involved in the damage of virally infected liver cells, both in acute and chronic hepatitis B (Edgington & Chisari, 1979; Alberti, *et al.*, 1977; Thomas *et al.*, 1982).

Although the 'over-all' patterns of viral antigenic expression and the inflammatory infiltrate are mutually related (Gudat *et al.*, 1975; Ray *et al.*, 1976), an *in situ* cell-to-cell relationship between the hepatocytes with viral antigenic expression and the lymphocytes involved in CMC or ADCC has not been established. In a previous paper (Eggink *et al.*, 1982a) we have reported on the lymphocyte subsets in CAH, both HBsAg positive and negative, especially in relation to the areas with liver cell necrosis. The purpose of this study was to demonstrate a relation between the liver cells with detectable expression of HBV antigens (HBsAg, HBcAg, or the 'e' antigen: HBeAg) and the subsets of mononuclear cells possibly involved in the immunological attack with special reference to differences in acute hepatitis B (AHB), chronic persistant hepatitis B (CPH-B) and chronic active hepatitis B (CAH-B).

MATERIALS AND METHODS

Patient groups. During the period 1979–1982, from a total number of 36 patients with fully documented HBV infection and a liver biopsy, 25 cases were included in this study. According to clinical and histological parameters the cases fitted into the following groups: AHB ($n=6$), CPH-B ($n=7$) and CAH-B with or without cirrhosis ($n=12$). Regular serum determinations of liver tests, HBV antigens, HBV antibody titres, immunoglobulin concentrations and antibody titres were performed in all cases of suspected or proven HBV infection according to protocol (Niermeijer & Gips, 1981). Some characteristics of the patient groups are given in Table 1.

An additional group of five cases with characteristic distribution patterns of the HBV antigens in the liver parenchyma served as a reference covering the whole range of possible expressions of HBV antigens in hepatocytes. This group consisted of cases with early AHB, CPH-B with 'ground glass' hepatocytes, CPH-B with only HBcAg expression in the liver, CAH-B with HBsAg

Table 1. Clinical data of the investigated groups of patients with hepatitis B

	AHB	CPH-B	CAH-B*
No. of patients	6	7	12
Age (Years)	20–69	30–55	25–47
Female/male	2/4	2/5	2/10
Duration of illness (months)	<1	>6	>6
Serum			
HBsAg/anti-HBs	6/—	7/—	12/—
anti-HBc IgM	6	7	12
HBeAg/anti-HBe	4/—	6/—	7/4*

AHB = acute hepatitis B; CPH-B = chronic persistent hepatitis B; CAH-B = chronic active hepatitis B.

* Including active cirrhosis.

† In one of the cases no HBeAg nor anti-HBe could be detected.

Between the groups with presence or absence of HBeAg in the serum and in the liver cell nuclei, no differences could be established in the activity of the inflammation nor in the relative contribution of the mononuclear subsets.

localization at cell membranes, and HBV infection in an orthotopic liver homograft with prominent expression of HBsAg and HBcAG at liver cell membranes.

The percutaneous liver biopsies were performed using a 1·6 mm Menghini needle. A small part of the biopsy was immediately frozen in freon-22 and stored at −70°C. The larger part of the biopsy was fixed in 4% paraformaldehyde with 5% glacial acetic acid and 6% $HgCl_2$, embedded in paraplast and used for light microscopy and some of the immunoperoxidase (IP) studies. For electron microscopy a very small part of the biopsy was fixed in phosphate-buffered 2% glutaraldehyde. Serial sections were cut from all specimens to enable the study of relations between all parameters in each case. For each method sections of all cases were simultaneously incubated together with positive controls.

HBV antigens in liver sections. HBsAg, HBcAg and HBeAg were demonstrated using peroxidase (PO) conjugated anti-HBs, anti-HBc and anti-HBe immunoglobulins, respectively. These antibodies were kindly provided after testing by Organon Scientific Development Group, Oss, The Netherlands. For HBsAg 6 μm paraffin and frozen sections were used, for HBcAg and HBeAg 6 μm frozen sections were used. After pre-incubation with normal human AB serum diluted 1:5 for 15 min at room temperature the sections were incubated with diluted anti-HBs ~ PO for 1 h at 37°C, and subsequently for the next 12 h at 4°C. Because the aHBe serum might show very weak anti-HBc reactivity, for the demonstration of HBeAg parallel sections were pre-incubated with normal human AB serum and subsequently with unconjugated anti-HBc serum for 1 h at 37°C and the next 12 h at 4°C. The anti-HBc serum was free of anti-HBe. Thereafter one section was incubated with anti-HBe ~ PO to demonstrate HBeAg, on the other section anti-HBc ~ PO was applied to test the blocking activity of pre-incubation with anti-HBc. The sections were stained with diaminobenzidin (DAB) for 5 min.

Lymphocyte subsets in liver sections. The lymphocyte subsets were characterized with hybridoma produced, monoclonal antibodies (MoAbs) which were obtained from Ortho Pharmaceutical Corporation (Raritan, New Jersey, USA) and the Leu antibodies from Beckton and Dickinson (Mountain View, California, USA). The production, growth and characterization of these antibodies have been the subject of a series of recent reports (Reinherz *et al.*, 1980; Ledbetter *et al.*, 1981; Verbi *et al.*, 1982; Kay & Horwitz 1980; Breard *et al.*,1980; Abo *et al.*, 1982).

To demonstrate reactivity of these lymphocyte subsets in tissue sections with MoAbs an indirect immunoperoxidase technique was used, as described in previous papers (Eggink *et al.*, 1982a, 1982b). In short, 6 μm thick frozen sections were air dried with ventilator for 30 min. The sections were fixed in acetone for 10 min at room temperature and shortly washed in PBS, pH 7·4 The sections were incubated with 25 μl of diluted antibody for 30 min. In addition, control sections were incubated with control ascitic fluid or PBS. All sections were subsequently incubated with peroxidase conjugated rabbit anti-mouse Ig serum (Dakopatts, Copenhagen, Denmark) diluted 1:20 for 15 min, supplemented with 1% human AB serum. Between incubations the sections were washed for 10 min in three changes of PBS. the sections were stained with 3-amino-9-ethylcarbazole and H_2O_2 during 10 min. Nuclear counterstaining was performed with Haemalaun and the sections were mounted with Aquamount (Gurr, Essex, UK).

Immunoglobulins and plasma cells in liver sections. Plasma cells and immunoglobulins at the surface of hepatocytes were demonstrated in 4 μm thick paraffin sections. After pre-incubation with normal rabbit serum diluted 1:15 at room temperature for 10 min, sections were incubated with peroxidase conjugated rabbit anti-human IgG, IgM or IgA serum (Dakopatts) for 60 min at 37°C. These sections were stained with DAB for 5 min and counterstained with Haemalaun. For every technique, control sections were incubated with normal serum or PBS in the first step.

Serological methods; additional to general protocol. The determinations of HBV antigens and antibodies in the serum of all patients in this study, taken at the time of liver biopsy, were repeated. HBsAg and a HBs were determined by solid phase radioimmunoassay (Abbot Diagnostic Division, North Chicago, USA), HBeAg and anti-HBe by enzyme immunoassay, anti-HBc total and anti-HBc-IgM, the latter as a marker of recent or ongoing HBV infection (Niermeijer *et al.*, 1978; Aldershvile *et al.*, 1981) were determined by enzyme immunoassay (Organon, Oss, The Netherlands).

RESULTS

The histology of the liver biopsies in each of the groups was fully in accordance with the diagnostic criteria for AHB, CPH-B and CAH-B, respectively (Bianchi *et al.*, 1977). In the reference group, the results with peroxidase conjugated antibodies to HBsAg, HBcAg and HBeAg were similar to those with unconjugated antibodies in an IF technique (Houthoff *et al.*, 1980). Furthermore, the presence and distribution of surface and core particles in EM closely correlated with the results of the IP and IF methods (Fig. 1).

In general, hepatocytes and portal tract structures were negative with all methods, apart from some immunoglobulin staining of the portal vessels and mesenchyme. The endothelial and Kupffer cells were negative with all methods, except for the Leu 3 antibody that showed weak staining of Kupffer cells. No $OKT6^+$ lymphocytes were found in any of the cases. The distribution in the liver of the mononuclear cell subsets (Fig. 2), characterized with the MoAbs, is summarized in Table 2.

In all cases of AHB some IgG positive plasma cells were present, mainly in the portal tracts. Immunoglobulins at the surface of hepatocytes were present in three cases, randomly distributed throughout the parenchyma. The main characteristics in AHB were the absence of demonstrable HBV antigens and the preponderance of non-T cells in the inflammatory infiltrate. In CAH-B plasma cells with IgG and IgA were scanty in most cases, plasma cells with IgM were absent. Immunoglobulins at liver cell membranes were present in five cases, mostly randomly distributed throughout the parenchyma and and not especially related to areas with PMN. At least one of the HBV antigens was present in hepatocytes in every case. In seven cases hepatocytes with a cytoplasmic HBsAg localization were found, their distribution and amount varied greatly from patient to patient, but also from area to area. Intranuclear HBcAg was found in randomly

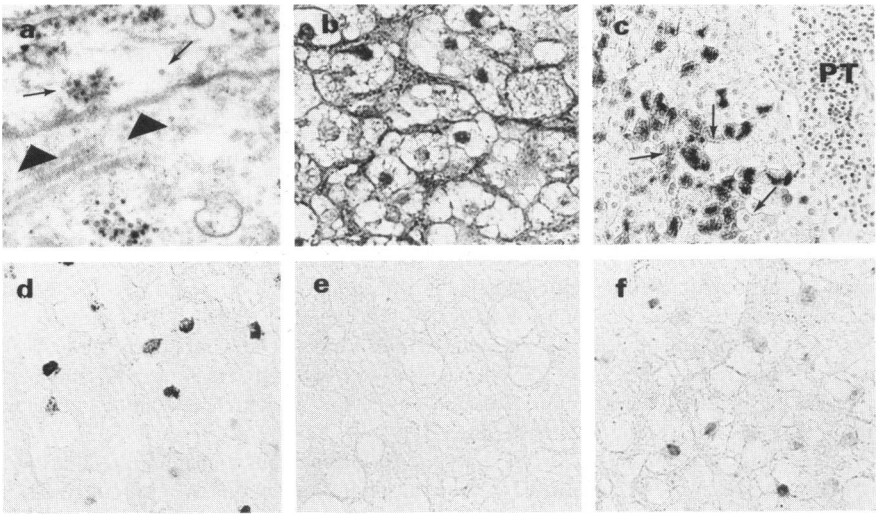

Fig. 1. Hepatitis B viral particles and the immunohistologic detection of viral antigens in the liver parenchyma. (a) Hepatitis B core particles (small arrows) in the cytoplasm of a hepatocyte near the plasma membrane (arrowheads) and in the adjacent extracellular space. Electron micrograph, magnification ×48,450. (b) Expression of HBcAg in the nuclei and plasma membranes of liver cells; to a lesser extent HBcAg is also present in the cytoplasm. anti-HBc ~ PO, magnification ×350. (c) Expression of HBsAg in the cytoplasm of many hepatocytes. Also, some HBsAg is present in relation to liver cell membranes (arrows). In a portal tract (PT) an inflammatory infiltrate is present with some spillover on the periportal parenchyma. Anti-HBs ~ PO with light haematoxylin counterstaining, magnification ×140. (d), (e) and (f) are from the same case and illustrate the differential detection of HBcAg and HBeAG, magnification ×350. (d) Incubation with anti-HBc ~ PO, showing HBcAg in liver cell nuclei. (e) Same, but pre-incubation with unconjugated anti-HBc. The HBcAg in the nuclei has been completely blocked. (f) Pre-incubation with unconjugated anti-HBc, incubation with anti-HBe ~ PO, showing HBeAg in liver cell nuclei.

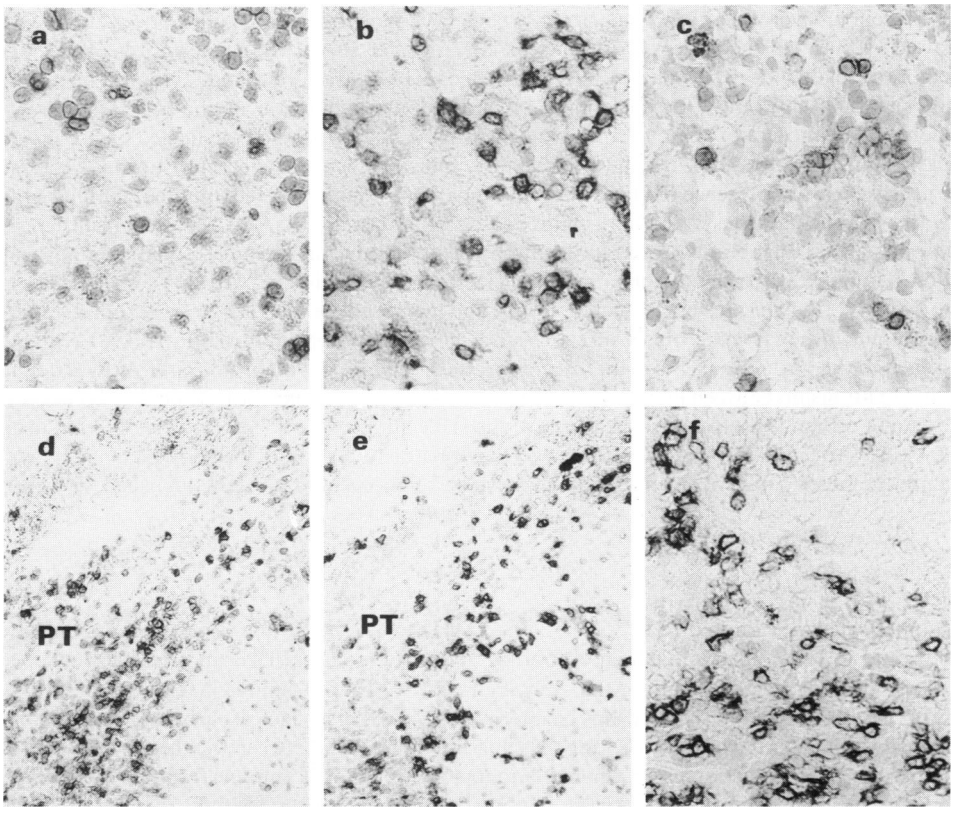

Fig. 2. Immunohistological demonstration of the phenotype of lymphocyte subsets in the liver. (a–c) Liver parenchyma in AHB (a) Leu 7^+, (b) $OKT8^+$ cells and (c) $OKT1^+$ cells. Note the relatively small number of pan T phenotypic cells in (c) as compared to the cells of suppressor/cytotoxic phenotype in (b) magnification × 350. (d & e) Consecutive sections of the same case, chronic persistent hepatitis B with some spillover of lymphocytes from the portal tract (PT) on the adjacent parenchyma. $OKT1^+$ cells (d) are mainly present in the portal tracts, whereas $OKT8^+$ cells (e) are mainly found in the periphery of the portal tracts and in the periportal parenchyma, magnification × 140. (f) CAH-B. $OKT8^+$ cells in a periportal area with piecemeal necrosis, magnification × 350.

Table 2. Distribution of the mononuclear cell subsets in liver biopsies of patients with AHB, CPH-B and CAH-B

		Liver parenchyma			Portal tracts		
Subsets	Functional interpretation	AHB	CPH-B	CAH-B	AHB	CPH-B	CAH-B
Leu 3^+	inducer/helper T cell	±	−	−	−	++	+
$OKT8^+$,11^+ $OKT1^+$,3^+	cytotoxic/suppressor T cell	±	±	++	+	+	+
$OKT8^+$,11^+ $OKT1^-$,3^-	(?) cytotoxic non-T cell	++	−*	−	−	−	−
Leu 7	NK cell	+	−	−	±	−	−
$OKM1^+$,2^+	K/NK cell	−	±	±	−	−	−
IgM^+	B cell	±	−	−	+	−*	−

− = absent, ± = some, + = appreciable number, ++ = many.
* Except for one case of otherwise proven CPH-B.

distributed hepatocytes of eight cases, in one case in combination with cytoplasmic HBsAg. HBeAg was present in seven cases. It was consistently and only found in the nuclei of hepatocytes, that also showed HBcAg, all the patients with nuclear HBeAg were also HBeAg seropositive. A liver cell membrane related localization of HBcAg, HBsAg or HBeAg was not found. The positive reaction with anti-HBc and anti-HBe was totally blocked by pre-incubation of the liver sections with unconjugated antisera of analogous titre and specificity, but it remained unaffected by pre-incubation with normal human sera. In the inflammatory infiltrate the main characteristic in CAH-B was the nearly exclusive presence of $OKT8^+$ T cells in parenchyma. In CPH-B immunoglobulins at the surface of the hepatocytes were found in three cases. Cytoplasmic HBsAg was present in all cases, in two cases also a membrane related localization was found. Of the eight cases with demonstrable HBcAg, in four cases nearly all hepatocytic nuclei were positive, in one case only some nuclei were positive, while in two cases nuclear, cytoplasmic and membrane related HBcAg was found. HBeAg was present in hepatocytic nuclei of six cases. The inflammatory infiltrate in CPH-B remained confined to the portal tracts, its main feature was the large proportion of Leu 3^+ T cells. A definite spatial relationship between liver cell necrosis and/or lymphocytic infiltration and/or a lymphocyte subset on one hand and the presence of one of the HBV antigens on the other was not found in any of the cases. In CAH-B the HBV antigens were less frequently found in the periportal parenchyma than in the other parenchymal zones. A negative correlation could not be established, however.

DISCUSSION

Uncomplicated, self limited AHB, CPH-B and CAH-B with or without cirrhosis represent three syndromes in HBV infection. The morphology and serology of CPH-B and CAH-B have been shown to occur transiently during the early course of typical self limited AHB (Houthoff *et al.*, 1980); the chronic conditions may thus be interpreted as the persistence of early stages of infection. The virus itself is not cytotoxic for liver cells and the differences between the three syndromes are supposed to depend at least in part upon different patterns of host's immune response. As shown in Table 2, there are indeed differences in the lymphocyte subsets between the three syndromes, both in the liver parencyma and to lesser extent in the portal tracts. The expression of HBV antigens on the liver cell surface has been postulated as the main target for the immunological reaction (Edgington & Chisari, 1975). In other viral hepatitis forms, e.g. in cytomegalovirus infection, a periportal or CAH type of hepatitis correlated with the presence of viral antigens in the periportal hepatocytes (Ten Napel, Houthoff & The, 1983). In a similar way, we tried to relate the mononuclear cell reaction to the presence of HBsAg, HBcAg and/or HBeAg in hepatocytes. In antigenic expression participated in the immune response, there should at least be a positive spatial correlation between the lymphocytic effector cells and the hepatocytes with demonstrable HBV antigens, this could not be demonstrated, however.

Membrane fixed immunoglobulins were an inconsistent finding, not related to areas with liver cell destruction, and although in agreement with the findings in other studies (Hopf *et al.*, 1975) do not seem to be involved in the pathogenesis either. In the serum, the presence of HBeAg or anti-HBe was not related to the mononuclear subsets involved, nor to the severity of the clinical or histological symptoms. For anti-HBe, this is in contrast to other reports (Eleftheriou *et al.*, 1975), but in general the serological findings are in accordance with other studies (Aldershvile *et al.*, 1981; Niermeijer *et al.*, 1978). An explanation of our findings might be that hepatocytes with viral replication more than those with expression of viral antigens are involved in the inflammatory response (Burrell *et al.*, 1982) and/or that other liver cell membrane antigens are also involved in the cytotoxicity reaction. *In vitro* studies with peripheral blood lymphocytes from patients with chronic HBV infection have shown cellular immunity to heterologous target cells coated with HBsAg (Warnatz *et al.*, 1979). Based on the the presence of the E rosette receptor it was concluded that these cells were T lymphocytes. However, it has been shown that the E rosette receptor is present both on T lymphocytes and on a population of non-T cells with NK or K activity (Verbi *et al.*, 1982). It also became clear that T lymphocytes as effector cells not only possess specificity for viral antigens but also require an additional recognition of HLA-A, B, C system (Dickmeiss, Soeberg & Sweygaard,

1977). Based on this data the effector cells in several *in vitro* studies were probable not T lymphocytes but NK or K cells. With MoAbs it is possible to define different subsets of T and non-T cells, both in peripheral blood and in the tissues. In a previous study (Eggink *et al.*, 1982) we reported that during CAH-B OKT8$^+$ cells of T cell origin (OKT1$^+$, 3$^+$, 11$^+$) were the predominant mononuclear cell type in the parenchyma of the liver and that these T cells were related to areas with liver cell necrosis. The results in this study corrobate this data. The HLA-A, B, C antigens are normally poorly expressed on hepatocytes but their expression is enhanced during viral infection and reactive conditions (Thomas *et al.*, 1982) As in the periportal areas of CAH-B these reactive circumstances are present and the presumably cytotoxic T cells are the only cell type found, it thus seems justified to conclude that T cell cytotoxicity under these circumstances is the only possible immune mechanism involved in the 'Piecemeal' necrosis of hepatocytes.

In CPH-B the main and characteristic features of the inflammatory infiltrate were the abundance of Leu 3$^+$ helper/inducer cells in the portal tracts and some mononuclear cells in the liver parenchyma. Immunological tolerance of the viral antigens or low expression of HLA antigens may be involved in CPH-B.

In AHB mononuclear subsets in the parenchyma were essentially of non-T, non-B cell origin, although some OKT8$^+$ T cells were also present. The two mainly occuring subsets were a population of NK cells (Leu 7$^+$, Abo *et al.*, 1982) and a population of OKT8$^+$ non-T cells (OKT1$^-$, 8$^+$, 11$^+$, Eggink *et al.*, 1982).

So far, the latter population has not been characterized functionally with *in vitro* methods, but its presence in areas of liver cell necrosis, both in AHB and in hepatitis of idiopathic autoimmune type, suggests a specific function in these syndromes. One of the possibilities might be that these OKT8$^+$ non-T cells are the effector cells in a non-T cell cytotoxic reaction, either as K cells in ADCC or as NK cells in CMC. The finding of Leu 7$^+$ cells in AHB is in agreement with the known occurrence of NK cells in other instances of acute immune reactions (Herberman *et al.*, 1982). The complete absence of OKM$^+$ cells in AHB is noteworthy in this context. Although Leu 7$^+$, OKT1$^-$. 3$^-$, 8$^+$, 11$^+$ and OKM$^+$ all specificy populations of non-T mononuclear cells with proven or presumed NK/K activity, these membrane antigens are not present on the same cell and appear to specify functionally different subsets.

In conclusion, our results demonstrate that (1) both T cells (OKT1$^+$, 3$^+$, 8$^+$, 11$^+$) and non-T cells (OKT1$^-$, 3$^-$, 8$^+$, 11$^+$, Leu 7$^+$) may be involved in a cytotoxicity reaction during hepatitis B infection; (2) the membrane markers for mononuclear non-T cells with potential or proved K/NK activity (OKT1$^-$, 3$^-$, 8$^+$, 11$^+$, Leu 7$^+$, OKM1$^+$, 2$^+$) specify different subsets; (3) the expression of Hbs/c/e Ag seems not to be directly involved in the immune reaction; (4) different immune mechanisms occur in AHB, CPH-B and CAH-B, with probably NK cell cytotoxicity in AHB and T cell cytotoxicity in CAH-B and (5) a defect in the activity of a population of NK cells (Leu 7$^+$) may be a major factor in the occurrence of CAH-B.

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