Liver and biliary

Cellular cytotoxicity against autologous hepatocytes in acute and chronic non-A, non-B hepatitis

T PORALLA, T H HÜTTEROTH, AND K-H MEYER ZUM BÜSCHENFELDE

From I Medizinische Klinik und Poliklinik, Johannes Gutenberg-Universität Mainz, Federal Republic of Germany

SUMMARY In a microcytotoxicity assay we tested lymphocyte cytotoxicity against autologous hepatocytes. The following cytotoxicity values were found (given mean $\pm$ SEM): acute non-A, non-B (NANB) hepatitis $45.7 \pm 4.3\%$ ($n=7$), chronic NANB hepatitis $32.8 \pm 5.1\%$ ($n=11$), chronic active hepatitis B (CAH-B) $27.7 \pm 6.7\%$ ($n=10$), toxic lesions $18.1 \pm 4.2\%$ ($n=18$), controls with normal liver histology or minimal changes $4.9 \pm 2.5\%$ ($n=8$). Thus our study shows enhanced cellular cytotoxicity in acute and chronic NANB hepatitis and indicates that T cells as well as non-T cells have cytotoxic effector functions. These findings are similar to those obtained in CAH-B and suggest that cellular immune reactions play an important role in the course of NANB hepatitis. For comparison we tested cytotoxic reactions in toxic lesions. They were only moderate and well distinguishable from those observed in NANB hepatitis and CAH-B; they even may be unspecific. No correlation was seen between cytotoxicity and aminotransferase concentrations.

There is convincing evidence now that the course of hepatitis B virus infections is determined by the host's immune reactions, mainly the cellular immune response¹ – nevertheless the target antigens and the effector cells for these reactions are still controversial. Therefore selection of an appropriate target cell system seems to be important. The use of autologous hepatocytes as targets²⁻⁷ hopefully will help to overcome some of the difficulties with allogeneic target systems such as differences in expression of alloantigens and other surface antigens.

Up until now enhanced cytotoxicity against autologous hepatocytes has been reported in small series of acute and chronic viral hepatitis,⁴⁻⁷ chronic active HBsAG-negative (presumably mainly auto-immune type) hepatitis⁵ and chronic active hepatitis B.²⁻⁶ Similar investigations in patients with liver diseases which are not believed to be mediated by immune reaction have not been reported, although they may help to establish the immunologic specificity of those phenomena. Until a detailed characterisation of viral antigens in NANB hepatitis will be available this autologous test system seems to

be the most promising one to study cellular immune reactions in NANB virus infection.

We therefore studied cellular cytotoxicity against autologous hepatocytes in patients with acute and chronic NANB hepatitis, chronic active hepatitis B and compared the findings with results in toxic lesions and normal controls.

Methods

PATIENTS

The following groups of patients were tested: (a) Seven patients with acute NANB hepatitis. (b) Eleven patients with chronic NANB hepatitis. Diagnosis in groups (a) and (b) was based on serological exclusion of hepatitis A and B, herpes, cytomegalo and Epstein-Barr virus infections. Anti-nuclear, antimitochondrial, and smooth muscle antibodies or liver membrane autoantibodies were not detectable and there was no history suggesting drug or alcohol induced liver damage. (c) Ten patients with HBsAG positive chronic active hepatitis. Six of them were HBeAG positive and the remaining four were HBeAG negative and anti-HBe positive. (d) Eighteen patients with alcohol induced toxic lesions. All these patients had a history of high alcohol consumption and did not show serological signs of viral or autoimmune type

Address for correspondence: Prof Dr K-H Meyer zum Büschenfelde, I Medizinische Klinik und Poliklinik, Johannes Gutenberg-Universität, Langenbeckstr 1, 6500 Mainz, Federal Republic of Germany.

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hepatitis. By histological examination fatty liver with or without fibrosis was found, chronic alcoholics with massive acute liver injury (so called alcoholic hepatitis) as well as cirrhotics were excluded. (e) Eight persons with normal liver histology or only minimal changes served as controls. So called healthy HBsAG carriers were excluded from this group.

This study includes only cases in which examination of the liver biopsy revealed a definite histological diagnosis. No one received immunosuppressive or antiviral therapy.

PREPARATION OF ISOLATED HEPATOCYTES

Isolation of the hepatocytes and cytotoxicity assay were performed according to Vergani⁵ with minor modifications. Approximately 3 mm of a liver biopsy specimen obtained for diagnosis or follow-up purposes were cut into small pieces and incubated for five hours in RPMI 1640 medium containing 10% fetal calf serum, 0.01% collagenase (Boehringer Mannheim, Germany), 2.5% hepes, streptomycin, penicillin, and amphotericin B adjusted to a pH of 7.35 in an atmosphere of 95% O₂ and 5% CO₂. After three washings with collagenase, free medium cells were counted and 100 hepatocytes were plated into each well of microculture plates (Falcon 3040) and incubated in an atmosphere of 95% O₂ and 5% CO₂ for 24 hours.

ISOLATION OF PERIPHERAL BLOOD LYMPHOCYTES

Twenty five millilitres of heparinised venous blood were incubated with Fe-dextran (Lymphocyte separating reagent, Technicon Instruments) for 30 minutes at 37°C to remove phagocytic cells. Thereafter mononuclear cells were separated over a Ficoll-Hypaque density gradient.⁸ Viability of the cells used exceeded 95% as determined by trypan blue dye exclusion. More than 95% were small lymphocytes. To obtain T cell enriched lymphocyte-fractions cells were incubated with sheep red blood cells previously coated with antibody and complement (EAC). After rosetting and centrifugation over a Ficoll-Hypaque gradient non-rosetting interface lymphocytes contained less than 2% EAC rosetting non-T cells. In the same way non-T cell enriched compartments were obtained by rosetting the T cells with sheep red blood cells pretreated with neuraminidase (Boehringer Mannheim, Germany) and centrifugation over a Ficoll-Hypaque gradient. Non-T cell enriched fractions contained less than 3% sheep red blood cell rosetting T cells. For some experiments heparinised blood was centrifuged over a Ficoll-Hypaque gradient without preincubation with Fe-dextran to obtain a cell suspension containing lymphocytes as well as macrophages.

MICROCYTOTOXICITY ASSAY

Twenty four hours after the incubation of the hepatocytes had been started the supernatant was aspirated from the wells and replaced by 10 µl of a lymphocyte suspension. At least 10 wells were filled with each of the lymphocyte preparations tested. Except for some experiments mentioned below effector/target cell ratio was 300:1. Adding medium without lymphocytes to 10–20 wells provided individual control values. After incubation for further 18 hours the plates were inverted for two hours and washed carefully, fixed with methanol, and stained with 1% eosin. Hepatocytes remaining in each well were counted and cytotoxicity was calculated according to the formula:

$$100 - \left(\frac{\text{number of cells in test wells}}{\text{number of cells in control wells}} \times 100 \right) = \% \text{ cytotoxicity}$$

Cytotoxicity assay was performed before diagnosis of the patient was known. Unlike Vergani³² we reduced the incubation period to 18 hours to minimise unspecific effects, as during this time T cell mediated as well as K and NK mediated cytotoxicity should have reached its maximum.⁹

STATISTICS

The exact Wilcoxon-Mann-Whitney test was used to compare two groups of patients.¹⁰ For calculation of significances we applied the Holm's procedure,¹¹ which simultaneously controls the error probability for all the tests used. As error probability we chose 5%. For some additional questions p values were calculated and interpreted descriptively.

Results

SPONTANEOUS LOSS OF HEPATOCYTES DURING THE INCUBATION PERIOD

At the end of the incubation period the number of hepatocytes remaining in the control wells was very similar in acute NANB hepatitis, chronic active hepatitis B as well as in the control group (Table 1).

Table 1 Number of hepatocytes remaining in individual control wells after incubation without lymphocytes (mean ± SEM)

Acute NANB hepatitis	107 ± 12
Chronic NANB hepatitis	93 ± 11
Chronic active hepatitis B	100 ± 12
Toxic lesions	63 ± 8
Normal or minimal changes	110 ± 21

In chronic NANB hepatitis hepatocytes incubated without lymphocytes were only slightly diminished (statistically not different from normal controls) while in toxic lesions there was a remarkable spontaneous loss of hepatocytes during the incubation period ($p < 0.05$ when compared with the controls). By electron microscopic studies 92–93% of the cells were found to be hepatocytes and 7–8% were Kupffer cells.

LYMPHOCYTE CYTOTOXICITY AGAINST AUTOLOGOUS HEPATOCYTES

Cytotoxic activity of non-separated lymphocytes against autologous hepatocytes was increased in patients with hepatitis NANB and B as well as in those with toxic lesions when compared with values obtained from persons with normal or minimally altered liver histology (Table 2, Figs. 1, and 2). There were distinct differences, though, between the diseases investigated: while in toxic lesions there was only a moderate increase in cytotoxicity which did not exceed mean plus two standard deviations of the control group ($p < 0.05$), the most striking rises in lymphocyte cytotoxicity were found in patients with acute NANB hepatitis and less in chronic NANB hepatitis. Cytotoxicity in both of them differed significantly from the control group ($p < 0.005$). In contrast the difference between acute and chronic NANB hepatitis was not statistically significant. In chronic active hepatitis B we found remarkably increased cytotoxicity ($p < 0.025$ when compared with the controls), less, though, than in NANB hepatitis.

Furthermore cytotoxicity in viral hepatitis was clearly greater than in toxic lesions ($p < 0.005$ in acute NANB hepatitis, statistically significant, $p < 0.025$ in chronic NANB hepatitis).

Table 2 Cellular cytotoxicity against autologous hepatocytes (mean $\pm$ SEM)

	<i>n</i>	Non-separated lymphocytes (%)	T cell enriched lymphocytes (%)	Non-T cell enriched lymphocytes (%)
Acute NANB hepatitis	7	45.7 $\pm$ 4.3	32.3 $\pm$ 10.6	21.2 $\pm$ 7.3
Chronic NANB hepatitis	11	32.8 $\pm$ 5.1	15.6 $\pm$ 4.7	22.3 $\pm$ 6.2
Chronic active hepatitis B	10	27.7 $\pm$ 6.7	23.3 $\pm$ 6.8	36.1 $\pm$ 8.4
Toxic lesions	18	18.1 $\pm$ 4.2	19.6 $\pm$ 4.1	28.4 $\pm$ 6.9
Normal or minimal changes	8	4.9 $\pm$ 2.5	9.5 $\pm$ 2.9	11.1 $\pm$ 3.9

Fig. 1 Lymphocyte cytotoxicity against autologous hepatocytes.

CYTOTOXIC ACTIVITY OF LYMPHOCYTE COMPARTMENTS

Cytotoxic activity was found in T cell enriched as well as in non-T cell enriched lymphocytes in all the diseases investigated. In most cases we found only minor differences between unseparated lymphocytes and T enriched or T depleted lymphocytes. In acute NANB hepatitis non-T cell enriched lymphocytes were less effective than T cell enriched fractions, while in the other groups non-T cell enriched fractions tended to have higher cytotoxic activity than T cell enriched lymphocytes (Table 2 and Fig. 3).

Fig. 2 Lymphocyte cytotoxicity against autologous hepatocytes (mean $\pm$ SEM).

Fig. 3 Cytotoxicity of lymphocyte compartments (mean $\pm$ SEM) $\square$ non-separated lymphocytes $\square$ T cell enriched lymphocytes $\blacksquare$ non-T cell enriched lymphocytes.

EFFECT OF MACROPHAGES AND MONOCYTES

In a number of cases cytotoxic activity of peripheral blood mononuclear cells including lymphocytes and macrophages and monocytes was compared with the effect of lymphocytes alone. While in chronic NANB hepatitis there was no additional cytotoxic effect of macrophages and monocytes detectable, in toxic lesions these effectors remarkably enhanced the cytotoxic reactions ($p < 0.01$, Table 3).

EFFECT OF DIMINISHED NUMBER OF EFFECTOR CELLS

While we usually used an effector/target cell ratio of 300:1 in some experiments we were able to titrate the effector/target cell ratio. A reduction of the number of lymphocytes led to a proportional decrease in cytotoxic activity, eventually reaching background level cytotoxicity seen in normal controls (Fig. 4).

CORRELATION BETWEEN CYTOTOXICITY AND AMINOTRANSFERASE CONCENTRATIONS

The cytotoxicity we found did not correlate with aminotransferase or bilirubin concentrations.

Table 3 Cellular cytotoxicity of lymphocytes and macrophages/monocytes against autologous hepatocytes

	Effector cells	
	Lymphocytes alone (%)	Lymphocytes and macrophages/monocytes (%)
Chronic NANB hepatitis (mean of n=5)	43	44
Toxic lesions (mean of n=5)	25	39

Fig. 4 Cellular cytotoxicity against autologous hepatocytes at various effector/target cell ratios (mean $\pm$ SEM).

Discussion

The use of autologous hepatocytes as target cells for studies of cellular cytotoxicity has some clear advantages and some disadvantages in comparison with the use of animal hepatocytes, cultured cell lines, or target cells coated with certain antigens. First this test system provides HLA identity between target and effector cells. There is increasing evidence now that this identity is a prerequisite for T cell cytotoxicity⁹⁻¹² based on findings in murine and human systems of MHC restricted cytotoxicity against virus infected target cells.¹³⁻¹⁵ Furthermore the use of autologous target cells offers the possibility to investigate the role of immune reactions in diseases, in which a detailed characterisation of the causative agent has not been available, yet, as in NANB hepatitis. The main disadvantage in the use of autologous target cells is the need of an enzymatic isolation of the cells and their reduced viability as determined by trypan blue dye exclusion. Nevertheless by electronmicroscopy the cells appear well preserved as Figure 5 shows, and they are able to synthesise albumin (G Mieli Vergani, personal communication). Even though these cells are not suitable for chromium release assays, comparative studies indicate a good correlation between microcytotoxicity assay and chromium release under similar experimental conditions.¹⁶ More detailed analysis revealed that in a microcytotoxicity assay some cells may still attach to the culture plates which do not satisfy other criteria of cell viability,¹⁷ so that the cytotoxicity measured in our study might rather be underestimated than overestimated. The low cytotoxicity values in our control group and the decline of cytotoxic activity with the reduction of the number of effector cells in patients with enhanced

Fig. 5 Electronmicroscopic appearance of an hepatocyte after isolation from a biopsy.

cytotoxicity both argue against cell detachment because of unspecific effects. Furthermore the similar number of hepatocytes counted in wells without lymphocytes in NANB hepatitis and chronic active hepatitis B as well as in controls seems to exclude the possibility of differences in intrinsic viability between these groups. Meanwhile other investigators have shown the applicability of similar experimental designs to the study of synovial membrane cells¹⁶ and thyroid cells.¹⁸

In NANB hepatitis the test system used in our study seems to be the most promising approach as long as detailed characterisation of NANB viral antigens and their detection on target cells are not available. Our study clearly shows a cellular cytotoxic activity similar to that found in chronic active hepatitis B in acute and chronic NANB hepatitis, suggesting that immunologic mechanisms are active in both diseases in spite of their histological differences especially the absence of dense lymphocytic infiltrations in NANB hepatitis which are common in chronic active hepatitis B.¹⁹

Our data further indicate that T cell enriched as well as non-T cell enriched lymphocyte compartments are able to mediate cytotoxic effects in acute and chronic NANB hepatitis as well as in chronic active hepatitis B. The latter finding is in agreement with observations from other authors, who used the same experimental design.^{2,6} Certainly there is more than one explanation for these findings. First

cytotoxic T cells as well as K cells (in the non-T cell enriched fractions) might operate in parallel, as suggested earlier.⁵ On the other hand NK cells which contaminate both T cell enriched and non-T cell enriched fractions^{20,21} might act as effector cells. This would fit their suggested role in virus infections.²² NK activity might at least in part be elicited by activated T cells.²³ Nevertheless while in chronic NANB hepatitis, as in chronic active hepatitis B, non-T cell enriched lymphocytes seem to be more effective, in acute NANB hepatitis T cell enriched fractions appear to be more important. This might indicate a change of the immunological mechanisms during the course of the disease. Similar changes have been suggested by others on the basis of a smaller number of patients.²⁴ Certainly many more studies are needed to gain more detailed insight into the pathogenesis of NANB hepatitis and to determine the pathogenetic relevance of the presumed direct cytopathic effect of the virus or viruses¹⁹ and the primary or secondary role of the immune system. Nevertheless our findings clearly indicate that cellular immune reactions are involved in the course of the disease. Unlike patients with toxic lesions we found no evidence in the small number of patients tested that macrophages or monocytes play an important role in the cytotoxic activity observed in NANB hepatitis.

In chronic active hepatitis B the enhanced cytotoxicity found confirms previous studies using autologous hepatocytes,^{2,4,6} cultured cell lines,²⁵⁻²⁸ rabbit hepatocytes,²⁹⁻³² and HBsAG-coated target cells.³³⁻³⁵

The cytotoxic reactions in patients with toxic lesions found in our study were easily distinguishable from those seen in patients with viral hepatitis and did not exceed mean plus two standard deviations of the controls. The cytotoxicity found in this group of patients probably reflects unspecific effects because of increased fat content or other toxic changes that could impair the ability of these cells to attach to the culture plates. The role of macrophages and monocytes which seem to enhance cytotoxic reactions in this disease is far from clear, as recent studies could show cytotoxic³⁶ as well as inhibitory effects of these cells.³⁷

In conclusion patients with acute and chronic NANB hepatitis exhibited enhanced cytotoxicity. The observation that T lymphocytes are the predominant effector cells in acute infection, whereas non-T cells are predominant in chronic hepatitis suggests that effector mechanisms in acute and chronic NANB hepatitis may be different.

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