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# Natural killer activity in patients with acute viral hepatitis

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## SUMMARY

Using the natural killer (NK) sensitive K562 cell line, enhanced NK cell cytotoxicity was demonstrable early in the course of acute hepatitis B while normal values were obtained in patients studied during convalescence. No evidence of enhanced NK activity was instead obtained in the course of acute non-A, non-B hepatitis. Serum levels of α-interferon, as determined by radioimmunoassay (RIA), were significantly increased in patients with acute hepatitis B showing enhanced NK cell activity but not in those with acute non-A, non-B hepatitis and normal NK cell activity. These results suggest that natural cytotoxicity may play a role early in the course of acute hepatitis type B, before antigen-specific T lymphocytes become fully operative.

**Keywords** natural killer activity viral hepatitis α-interferon

## INTRODUCTION

The cytotoxic effect of peripheral blood mononuclear cells (PBMNC) has been extensively evaluated during viral hepatitis (Dienstag, 1984) and evidence has been obtained that virus-specific cytotoxic T lymphocytes may have pathogenetic relevance in hepatitis B, by reacting with the hepatitis B core antigen, on the surface of infected hepatocytes (Mondelli *et al.*, 1982; Trevisan *et al.*, 1981). Other lymphocyte populations, however, may also have a role in the elimination of infected hepatocytes, including the natural killer (NK) cell population which is involved in host resistance to viral infections (Herberman & Ortaldo, 1981).

Studies on NK activity have been conducted in chronic HBV infection and the results, obtained using different types of target cells, have been controversial (Dienstag & Bhan, 1980; Chisari *et al.*, 1981; Hutteroth *et al.*, 1982). Few data have been reported on NK cell activity in acute hepatitis B virus (HBV) infection, when the role of these effectors could be of greater relevance. We have therefore investigated NK cell activity in PBMNC in acute hepatitis type B, using a standard cytotoxicity assay. A group of patients with acute non-A, non-B hepatitis was included for comparison. Serum levels of α-interferon, whose production is closely related to NK activity, were also measured in all patients by radioimmunoassay.

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## MATERIALS AND METHODS

*Patients.* PBMNC were obtained from 25 patients during the course of acute, self-limited, hepatitis type B (11 males & 14 females, median age 23 years, range 19–43 years).

All patients presented with jaundice and with a five-fold or higher increase in alanine aminotransferase (ALT) levels. All were HBsAg positive and IgM anti-HBc positive during acute phase and became HBsAg negative during convalescence. The duration of illness at the time of testing in individual cases was defined as days after they had become jaundiced. None of the 25 patients were anti-delta positive in serum during acute phase by RIA.

Eleven additional patients (7 males & 4 females, median age 21 years, range 18–30 years) with acute hepatitis who were HBsAg, IgM anti-HBc, IgM anti-HAV and IgM anti-CMV negative in serum (acute non-A, non-B hepatitis by exclusion criteria) were studied 3 to 20 days after clinical onset. The most likely source of infection in these 11 patients was parenteral exposure due to drug addiction. Fourteen healthy subjects (7 males & 7 females, median age 23 years, range 18–41 years) were studied as the control group.

*Target cells.* The NK-sensitive K562, a continuous cell line derived from a patient with erythroleukaemia in blast crisis (Lozzio & Lozzio, 1975) were maintained in RPMI-1640 medium (GIBCO, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS, Difco), 2 mM L-glutamine, 20 mM HEPES buffer, 200 iu/ml penicilin, 100 $\mu$g/ml streptomycin and 2 $\mu$g/ml amphotericin B (complete medium). $1 \times 10^6$ target cells were incubated with 100 $\mu$Ci of $Na_2{}^{51}CrO_4$ in 1 ml of complete medium at 37°C for 2 h with occasional gentle agitation. Labelled cells were subsequently washed exhaustively to remove free radioactivity and resuspended at a concentration of $1 \times 10^5$/ml of complete medium.

*Effector cells.* PBMNC were prepared from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation (Bøyum, 1968), washed three times in balanced salt solution and adjusted to a concentration of $8 \times 10^6$ viable cells/ml.

*Cytotoxicity assay.* Triplicate cultures of $1 \times 10^4$ labelled target cells per well were incubated with PBMNC in sterile V-bottomed microtitre plates (Titerteck, Falcon Products, Becton Dickinson, Cockeysville, MD, USA) at two-fold decremental effector to target cell effector/target (E/T) ratios ranging from 80:1 to 10:1. Plates were than centrifuged at 100 *g* for 5 min and incubated at 37°C for 4 h in a humidified atmosphere of 5% $CO_2$ in air. After incubation, the plates were centrifuged again, 100 $\mu$l aliquots of supernatants harvested and released radioactivity detemined in a gamma counter. Total releasable counts were obtained by incubating target cells with complete medium containing 0·1% nonionic detergent (NP40). Spontaneous release was determined by incubating target cells with complete medium alone. Spontaneous isotope release ranged from 4 to 7%.

Cytotoxicity was expressed as follows:

$$\text{Specific lysis (\%)} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Total release} - \text{spontaneous release}} \times 100$$

*Serological tests.* HBsAg, HBeAg and anti-HBe, IgM anti-HBc, IgM anti-HAV and anti-delta were tested in serum by commercial RIA kits (Abbott Laboratories, Irving, TX, USA). IgM anti-CMV were tested by enzyme-linked immunosorbent assay (ELISA) (Behring Institute, Behringwerke, Marburg, FRG).

*Serum levels of* $\alpha$-interferon. Serum $\alpha$-interferon was measured by RIA (Interferon Kit, Dainabot Co., Ltd.) according to the manufacturer's instructions. Using the standard calibration curve provided with the kit, serum levels of $\alpha$-interferon in 14 healthy control subjects were $71 \pm 14$ (mean iu/ml $\pm$ s.d.).

*Statistical analysis.* The non-parametric Mann–Whitney U test for unpaired data was employed for the evaluation of the statistical significance of the difference in cytotoxicity found in the experiments. The relationship between percentage cytotoxicity and aminotransferase levels was assessed by the Spearman's rank correlation test.

## RESULTS

*NK cytotoxicity in acute hepatitis B*

The values for NK cell activity in each individual patient with acute hepatitis type B are given in Table 1 in relation to the time of study after clinical onset (jaundice), transaminase levels and HBeAg/anti-HBe status. Values for α-interferon in serum are also given in the table. Mean NK cell activity levels at different E/T ratios in healthy controls and in patients with acute hepatitis B and non-A, non-B are given in Table 2.

**Table 1.** NK cell activity at different phase of acute hepatitis B in individual patients and transaminase levels, HBeAg/anti-HBe status and α-interferon levels at the time of testing

| Patient N° | Days after jaundice | Cytotoxicity (%) 80:1* | 40:1 | 20:1 | 10:1 | ASL/ALT† levels (iu/ml) | HBeAg/antiHBe† status | α-IFN† levels (iu/ml) |
|---|---|---|---|---|---|---|---|---|
| 1 | 2 | 76 | 64 | 56 | 38 | 2100/3800 | $E^+$ | 123 |
| 2 | 1 | 65 | 50 | 34 | 16 | 92/300 | $E^-$ | 79 |
| 3 | 2 | 66 | 46 | 29 | 16 | 1850/3600 | anti-$E^+$ | 109 |
| 4 | 1 | 65 | 51 | 38 | 25 | 1000/2600 | $E^+$ | 120 |
| 5 | 2 | 72 | 51 | 29 | 14 | 670/1560 | $E^+$ | 78 |
| 6 | 4 | 65 | 52 | 40 | 26 | 940/2800 | anti-$E^+$ | 85 |
| 7 | 3 | 57 | 39 | 25 | 12 | 603/710 | $E^+$ | 111 |
| 8 | 1 | 65 | 60 | 54 | 40 | 1052/809 | $E^+$ | 96 |
| 9 | 1 | 51 | 43 | 24 | 14 | 610/1710 | E/anti-$E^-$ | 99 |
| 10 | 1 | 47 | 32 | 18 | 13 | 1205/2150 | $E^+$ | 88 |
| 11 | 2 | 91 | 84 | 58 | 32 | 663/2046 | anti-$E^+$ | 97 |
| 12 | 25 | 50 | 40 | 29 | 15 | 60/82 | $E^+$ | 106 |
| 13 | 30 | 53 | 34 | 25 | 15 | 70/260 | anti-$E^+$ | 69 |
| 14 | 21 | 36 | 28 | 19 | 12 | 650/3100 | anti-$E^+$ | 75 |
| 15 | 25 | 32 | 22 | 18 | 14 | 1120/1220 | $E^+$ | 69 |
| 16 | 28 | 36 | 26 | 19 | 10 | 266/437 | anti-$E^+$ | 54 |
| 17 | 21 | 29 | 24 | 17 | 8 | 375/579 | anti-$E^+$ | 67 |
| 18 | 22 | 51 | 29 | 15 | 8 | 1009/1267 | $E^+$ | 70 |
| 19 | 23 | 36 | 24 | 18 | 10 | 141/552 | anti-$E^+$ | 66 |
| 20 A | 3 | 69 | 56 | 37 | 23 | 3400/3100 | $E^+$ | 113 |
| 20 B | 21 | 44 | 25 | 10 | 8 | 420/1800 | E/anti-$E^-$ | 65 |
| 21 A | 1 | 67 | 43 | 26 | 17 | 2600/2200 | anti-$E^+$ | 83 |
| 21 B | 24 | 81 | 58 | 32 | 17 | 1800/1200 | anti-$E^+$ | 61 |
| 21 C | 32 | 60 | 45 | 32 | 15 | 106/280 | anti-$E^+$ | 51 |
| 22 A | 3 | 75 | 56 | 41 | 26 | 1650/2100 | $E^+$ | nt |
| 22 B | 12 | 55 | 43 | 29 | 18 | 850/1100 | $E^+$ | nt |
| 22 C | 23 | 42 | 32 | 25 | 14 | 130/350 | anti-$E^+$ | nt |
| 23 A | 2 | 65 | 51 | 38 | 23 | 1000/2800 | $E^+$ | nt |
| 23 B | 10 | 48 | 36 | 27 | 15 | 750/1260 | anti-$E^+$ | nt |
| 23 C | 21 | 30 | 24 | 16 | 10 | 266/436 | anti-$E^+$ | nt |
| 24 A | 2 | 79 | 61 | 46 | 31 | 2700/3350 | $E^+$ | nt |
| 24 B | 10 | 46 | 38 | 28 | 16 | 1100/1600 | $E^+$ | nt |
| 24 C | 21 | 43 | 31 | 22 | 13 | 460/690 | $E^+$ | nt |
| 25 A | 4 | 58 | 41 | 29 | 19 | 1250/740 | anti-$E^+$ | nt |
| 25 B | 13 | 36 | 25 | 15 | 9 | 560/380 | anti-$E^+$ | nt |

* E/T ratio.

† These data were obtained at the time of NK cell activity testing.

nt Not tested.

**Table 2.** NK cell activity in acute hepatitis B and non-A, non-B

| Subjects | N° Cases | Significance *v* controls | Cytotoxicity (%) (mean ± s.e.m.) 80:1 | 40:1 | 20:1 | 10:1 |
|---|---|---|---|---|---|---|
| Controls | 14 | — | 38·9 ± 3·6 | 28·6 ± 2·9 | 17·1 ± 2·0 | 9·7 ± 1·4 |
| Acute hepatitis Type B | | | | | | |
| All cases tested | 35 | $P < 0{\cdot}01$ | 56·8 ± 3·3 | 42·7 ± 3·1 | 29·2 ± 2·6 | 17·4 ± 1·8 |
| Early phase | 19* | $P < 0{\cdot}01$ | 65·8 ± 3·0 | 51·6 ± 3·5 | 36·0 ± 3·5 | 22·0 ± 2·6 |
| Recovery phase | 16* | $P$ = ns | 46·1 ± 4·5 | 32·2 ± 3·3 | 21·2 ± 2·1 | 12·0 ± 1·0 |
| Acute hepatitis Non-A, non-B | 11 | $P$ = ns | 46·6 ± 6·9 | 34·5 ± 5·9 | 22·0 ± 4·6 | 13·4 ± 2·9 |

*$P < 0{\cdot}01$; ns Not significant.

Patients with acute hepatitis B showed enhanced cytotoxicity of PBMC for K562 target cells at all E/T ratios, when compared with control subjects (Table 2). When patients were divided according to the phase of illness, NK cytotoxicty was significantly increased during the first 10 days after clinical onset but did not differ from controls during convalescence (Table 2).

The results of sequential estimation of NK activity in individual patients were in agreement with these data, as shown in cases 20, 21, 22, 23, 24, 25 of Table 1 in whom a progressive reduction in percentage cytotoxicity was observed at all E/T ratios from early phase of illness down to the convalesence phase. As can be seen in Table 1, there was no correlation between cytotoxicity values and aminotransferase levels ($r = 0{\cdot}1$) or HBeAg/anti-HBe status.

*NK cytotoxicity in acute non-A, non-B hepatitis*

As shown in Table 2, cytotoxicity for K562 target cells during acute non-A, non-B hepatitis did not differ from that found in the control group, although patients with non-A, non-B hepatitis were comparable to those with hepatitis B with respect to frequency and type of clinical symptoms and to peak aminotransferase levels.

*α-Interferon levels in serum*

Serum samples from patients with acute hepatitis B and non-A, non-B, obtained at the time their lymphocytes were studied for NK activity, were tested for α-interferon by RIA.

Mean serum levels in a group of healthy controls were 71 ± 14 iu/ml (mean ± s.d.). Twenty-one of the 25 patients with acute hepatitis B were tested and serum α-interferon levels were significantly higher (98 ± 15 iu/ml, $P < 0{\cdot}01$) than those of the control group in samples obtained early in the course of infection, at the time of increased NK cell activity, but not those obtained at later stages of the illness (68·7 ± 14 iu/ml, $P$ = ns). The difference in NK cell activity between these two phases of illness was also statistically significant ($P < 0{\cdot}01$).

Mean serum α-interferon values in patients with acute non-A, non-B hepatitis (70·9 ± 12 iu/ml) were similar to those of the control group.

## DISCUSSION

In the present study, we have investigated NK cell activity in viral hepatitis using the K562 cell line since NK cells lysing virus infected target cells have the same phenotypic and morphological characteristics of those active against K562 target cells (Perussia, Fanning & Trinchieri, 1982; Santoli, Trinchieri & Lief, 1978; Zarling & Yasukawa, 1983). Enhancement of natural cytotoxicity

was demonstrable early in the course of acute hepatitis B, while NK cell activity returned to normal later during convalescence, in a manner similar to the kinetics of the cytotoxic response in influenza virus infection (McMichael *et al.*, 1983).

Despite the enhancement of NK cell activity, levels of interferon in the serum were slightly elevated in the early phase of illness compared to values observed in healthy controls. These results appear to be at variance with those of a previous study in patients with acute hepatitis B, in whom serum levels of α-interferon, as measured by biological assay and radioimmunoassay, were approximately 10 times the upper limit of normal (Levin & Hahn, 1982).

One possible explanation for these discrepancies could be that interferon is increased in serum only in the pre-clinical or very early clinical phase of the disease, when viral replication is at its acme, while the effect of interferon on NK cells could persist for longer. Some evidence in support of this hypothesis comes from preliminary data in patients with acute hepatitis B, showing increased serum levels of interferon (by RIA and biological assay) in the pre-clinical phase of the disease, which rapidly returns to normal at clinical onset (H. C. Thomas, personal comunication). In fact, although the difference in mean, α-interferon levels between the early and late phase of acute hepatitis B in our patients was modest, nevertheless it was statistically significant, suggesting that interferon levels could have been more elevated during the incubation period and were returning to normal at the time we first tested our patients soon after clinical presentation.

On the basis of these functional studies, natural cytotoxicity may be one mechanism that controls the viral infection before antigen-specific cytolytic T cells become fully operative and may have some role in limiting the phase of virus multiplication. Support for this hypothesis comes from phenotypic analysis of the lymphoid cell infiltrate in the liver of patients with acute hepatitis B. This shows an increased proportion of NK cells (Eggink *et al.*, 1984), whilst suppressor/cytotoxic T cells usually predominate in the liver during chronic infection (Eggink *et al.*, 1984). The hypothesis that NK cells may also have a role in provoking the liver cell damage is not supported by our results since no relationship was found between NK cell cytotoxicity and ALT values.

We found no evidence of enhanced NK cell activity in acute non-A, non-B hepatitis, suggesting that the non-A, non-B agent(s) may be poor NK cell inducer, similar to meningitis virus infection in mice (Welsh & Kiessling, 1980).

Preliminary evidence suggest that at least some of the agents responsible for non-A, non-B hepatitis may belong to the retrovirus group, and low NK cell activity has been described in retroviral infections (Lopez, Kirkpatrick & Fitzgerald, 1982). Whether the lack of NK cell response plays any role in the progression to chronicity, that is frequently observed in non-A, non-B hepatitis (Realdi *et al.*, 1982), remains to be established.

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