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Interference of hepatitis B virus surface antigen with natural killer cell function

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SUMMARY

The influence of purified hepatitis B virus surface antigen (HBsAg) preparations or of supernatants derived from PLC/PRF/5 cell line (which produces HBsAg) on human natural killer (NK) activity was examined. Lymphocytes pre-incubated with HBsAg and subsequently washed showed a significant decrease in NK cytotoxicity against K-562 target cells. This effect was reversible and dose-dependent. In addition, pre-incubation with either HBsAg or PLC/PRF/5 supernatants inhibited in a reversible manner lymphocyte–K-562 conjugates and the binding of B73.1 monoclonal antibody (MoAb), which recognizes Fc receptors on NK cells. This effect was not observed with HNK-1, T3, T4, T6, T8 and T11 MoAb. HBsAg was non-toxic to lymphocytes, and ineffective with K-562 target cells. β-interferon did not modify HBsAg-mediated inhibition, when added either before or during the contact with HBsAg. Moreover, no modification was observed when neutrophils (at various neutrophil:lymphocyte ratios) were added, even though HBsAg is known to stimulate neutrophils to produce oxygen radicals which may modulate NK activity. We speculate that HBsAg produces these effects by reacting into receptor sites (possibly Fc receptor sites) on NK cell membrane. The overall significance of our results in relation to hepatitis and hepatocellular carcinoma is discussed.

Keywords natural killer cell function liver disease hepatitis B virus β-interferon

INTRODUCTION

Natural killer (NK) cells, which display spontaneous cytotoxicity to virus infected cells, have been proposed as mediators of resistance to some virus infections in man (Biron & Welsh, 1982). At the present there is no firm evidence that NK cells may perform a similar function in hepatitis B virus (HBV) infection. In most reports, a non-specific enhancement of NK activity was found (Dienstag & Bhan, 1980; Dienstag, Savarese & Bhan, 1982). In addition, Chin *et al.* (1983) reported that NK cells from convalescent hepatitis B subjects were specifically cytotoxic against Alexander PLC/PRF/5 cell line, which naturally expresses and secretes HBV surface antigen (HBsAg). In contrast, Keong, Herman & Rabson (1983) have recently reported that supernatants derived from this same cell line are able to depress *in vitro* NK cell activity.

In the present study we demonstrate that this effect may be produced by HBsAg, by blocking the binding of NK cells to target cells. Our experiments indicate that HBsAg-mediated depression is dose-dependent, reversible, unaffected by β-interferon (IFN-β) or neutrophils (PMN), and associated with a block in Fc receptors (FcR) or structures closely related to them. These findings

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may have pathogenetic implications in HBsAg positive chronic hepatitis and possibly in hepatocellular carcinoma.

MATERIALS AND METHODS

Peripheral blood leucocytes (PBL). PBL were obtained from 14 healthy donors (personnel from our laboratory or medical students, aged 21–34 years). Lymphocytes were prepared by centrifugation on Ficoll-Isopaque gradient (density 1·077 g/l) and depleted of adherent cells by two consecutive incubations (45 min at 37°C) on Petri culture dishes (Falcon products, Oxnard, California, USA). This procedure yields lymphocyte suspensions containing less than 2% contaminating monocytes based on morphology and peroxidase staining. PMN were obtained from the upper two-thirds of the plasma, after sedimentation of erythrocytes at 37°C for 45 min with 6% dextran (Pharmacia Fine Chemicals, Inc., Piscateway, New Jersey, USA) in saline. Contaminating erythrocytes were lysed by treatment with hypotonic medium (97% or more of the cells were morphologically PMN, as judged by Wright's staining).

NK cytotoxicity assay. NK cell activity was tested in a 4 h assay, set up in triplicate, according to the method described by West *et al.* (1977). K-562 leukaemic cells free from mycoplasma contamination (kindly provided by Dr G. Trinchieri, the Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania, USA) were used as targets. Unless otherwise indicated, results were quantitated at 50:1, 25:1, 12.5:1, and 6.25:1 effector (E) to target (T) cell ratios (E:T), and expressed as percentage cytotoxicity (% cytotoxicity) which was calculated as described by Keong *et al.*, 1983.

Lymphocyte–K-562 conjugates. Lymphocyte–K-562 cell conjugates (Herberman *et al.*, 1980) were obtained by mixing equal numbers of lymphocytes and K-562 cells. The cell mixture was spun (200*g* for 3 min) and incubated at 37°C for 2 h. Subsequently the pellet was gently resuspended, stained with Turk's solution and examined by light microscopy. The percentage of K-562 cells with three or more lymphocytes bound to them was quantitated after 200 K-562 cells had been counted.

MoAb. B73.1 MoAb, generously supplied by Dr G. Trinchieri, which recognizes FcR on antibody-dependent killer (K) and NK cells (Perussia *et al.*, 1983) was used. In addition, HNK-1 (Becton-Dickinson, Sunneyvalle, California), T3, T4, T6, T8 and T11 (Ortho Pharmaceutical Corp., Raritan, New Jersey) MoAb were used. HNK-1 MoAb has been described by Abo & Balch (1981) as specific for K and NK cells, but the HNK-1(+) subset coincides only partially with the B73.1(+) subset (Perussia *et al.*, 1983). The MoAb of T series recognizes lymphocyte subsets in which the whole T population, helper T cells, common thymocytes, cytotoxic suppressor T cells and E rosette forming cells are thought to be respectively included (Aiuti *et al.*, 1983). Indirect immunofluorescence was used to assess the binding of MoAb, as we have previously described (Aiuti *et al.*, 1983).

Pre-treatment of lymphocytes with PLC/PRF/5 culture supernatants. Lymphocyte–K-562 conjugates and studies with MoAb were carried out after 5×10^6 lymphocytes had been incubated (6 h at 37°C) in either fresh culture medium or supernatants derived from PLC/PRF/5 cell line (a gift from Dr I. Millman, Institute for Cancer Research, Philadelphia), as previously described (Keong *et al.*, 1983). Control experiments were carried out pre-treating lymphocytes with supernatants derived from Chang liver cell line, which does not produce hepatitis B virus surface antigen (HBsAg) (Dienstag & Bhan, 1980). In addition, experiments were carried out pre-treating K-562 cells with PLC/PRF/5 supernatants, to exclude any effect on these cells. Lymphocytes were washed three times in RPMI 1640 medium (Eurobio, Paris) before use.

Pre-treatment of lymphocytes with HBsAg purified preparations. A purified formalin free 22 nm HBsAg particle preparation ($2{\cdot}59 \times 10^{11}$ particles/μg) was kindly given us by Institut Pasteur, Paris (Batch 82). In order to assess the putative inhibitory effect of HBsAg, 5×10^6 lymphocytes were preincubated (for 60 min at 37°C) in 200 μl of RPMI 1640 medium either alone or with the addition of 12·5 μg HBsAg. At this concentration, the number of 22 nm particles per cell ($6{\cdot}4 \times 10^5$/cell) was within the range which is seen in peripheral blood of infected individuals, where up to 10^{13} particles/ml may be present (Mims & White, 1984). NK cytotoxicity assays, and studies with

lymphocyte–K-562 conjugates and with MoAb were carried out after lymphocytes were washed three times in RPMI 1640 medium. In parallel experiments, lymphocytes were tested after a second 24 h incubation (at 37°C in a humidified 5% CO_2/95% air atmosphere) in RPMI 1640 medium to which was added 10% fetal calf serum (GIBCO Europe Limited, Middlesex, UK). Finally, NK cytotoxicity assays were carried out also on lymphocytes pre-treated with 25 and 6·25 μg HBsAg/5 × 10^6 cells (E:T = 25:1) to assess whether the putative HBsAg-mediated inhibition depended upon the dose of HBsAg. Control experiments were performed to exclude any effect of HBsAg on K-562 cells.

Experiments in the presence of neutrophils. To assess the effect of purified PMN preparations, PMN and 12·5 μg HBsAg were incubated with lymphocytes throughout the cytotoxic assay. PMN and 1,000: u/ml of IFN-β as previously described (Trinchieri, Perussia & Santoli, 1980). The human IFN-β (1·8 × 10^6 iu/ml; 0·5 mg protein/ml) was kindly provided by the Sclavo Institute, Sienna, Italy. Lymphocytes were washed three times in RPMI 1640 medium before performing cytotoxicity

Treatment with IFN-β. Five million lymphocytes were incubated for 18 hours (at 37°C in a humidified 5% CO_2/95% air atmosphere) in RPMI 1640 medium containing 10% fetal calf serum and 1,000 iu/ml of IFN-β as previously described (Trinchieri, Perussia & Santoli, 1980). The human IFN-β (1·8 × 10^6iu/ml; 0·5 mg protein/ml) was kindly provided by the Sclavo Institute, Siena, Italy. Lymphocytes were washed three times in RPMI 1640 medium before performing cytotoxicity assays, lymphocyte–K-562 conjugates and studies with MoAb. In another set of experiments, 12·5 μg of HBsAg were added throughout the incubation with IFN-β.

Viability tests. The viability of lymphocytes incubated with either PLC/PRF/5 supernatants or HBsAg was assessed by Trypan Blue exclusion. It was always greater than 94%.

HBV serum markers. HBsAg and anti-HBs antibodies were tested in serum samples from the donors, using radioimmunoassay techniques (AUSRIA and AUSAB, respectively, Abbott Lab. North Chicago, Illinois, USA).

Statistical methods. The results were expressed as mean ± s.d., and evaluated statistically by Student's *t*-test (after angle transformation of data expressed as percentages) or by analysis of variance and subsequent Tukey's test (Lison, 1958).

RESULTS

NK cytotoxicity assays

Pre-incubation of lymphocytes with HBsAg resulted in a significant decrease at all E:T ratios ($P < 0.01$) in NK cytotoxicity, as shown in Fig. 1. Fig. 1 shows also that lymphocytes regained their original cytolytic activity after a 24 h recovery period. In addition, the degree of HBsAg-mediated inhibition was dose-dependent, as shown in the dose–response curve illustrated in Fig. 2. Normal results were obtained in control experiments carried out with HBsAg pre-treated K-562 target cells.

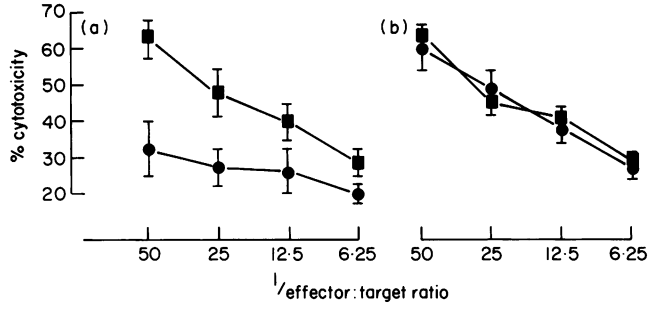

Fig. 1. NK cell activity in lymphocytes pre-treated with HBsAg (12.5 μg/5 × 10^b lymphocytes). (a) Lymphocytes pre-incubated (1 h) in medium (■) or HBsAg (●) and tested immediately. (b) Lymphocytes pre-incubated (1 h) in medium (■) or HBsAg (●) and tested after a second 24 h incubation in medium.

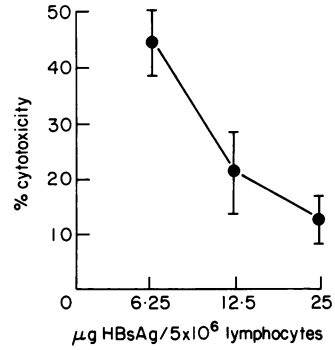

Fig. 2. Dose–response curve of HBsAg-mediated inhibition of NK cytotoxicity (effector:target ratio 25:1). Results obtained with different HBsAg concentrations differed significantly ($P<0.01$).

Lymphocyte–K-562 conjugates and reaction with MoAb

Pre-incubation with either PLC/PRF/5 supernatants or HBsAg resulted in a significant decrease in lymphocyte–K-562 conjugates and B73.1(+) cells (Table 1). On the contrary, PLC/PRF/5 supernatants or HBsAg did not modify the percentages of HNK-1(+), T3(+), T4(+), T6(+), T8(+) and T11(+) cells. PLC/PRF/5 supernatants- and HBsAg pre-treated lymphocytes showed normal values of lymphocyte–K-562 conjugates and B73.1(+) cells after a 24 h recovery period (Table 1). Pre-incubation with supernatants derived from cultures of Chang liver cells did not modify the percentages of lymphocyte–K-562 conjugates and B73.1(+) cells. PLC/PRF/5 supernatants or HBsAg pre-treated K-562 cells gave rise to normal values of lymphocyte–K-562 conjugates.

Table 1. Lymphocyte–K-562 conjugates and reactivity with B73.1 MoAb (mean ± s.d. of 14 experiments) in PLC/PRF/5 supernatants or HBsAg pre-cultured lymphocytes. Results obtained after a 24 h recovery period are reported in parentheses

	Lymphocyte pre-cultured with			
	Medium	PLC/PRF/5 supernatants	HBsAg	*P*
Conjugates %	15·4 ± 2·7	10·8 ± 3·1	7·9 ± 2·3	< 0·05
	(15·2 ± 3·3)	(14·9 ± 5·6)	(14·0 ± 6·3)	Not significant
B73.1(+) cells%	14·4 ± 2·7	11·8 ± 5·1	7·9 ± 2·3	< 0·05
	(13·9 ± 4·4)	(13·1 ± 4·7)	(12·9 ± 5·4)	Not significant

Effect of the presence of neutrophils

NK activity was tested in the presence of either HBsAg alone or HBsAg plus PMN (at various PMN:lymphocyte ratios) throughout the cytotoxic assay. In both cases NK activity did not differ from that observed on HBsAg pre-treated lymphocytes. PMN and HBsAg did not affect K-562 target cells.

Effect of the presence of IFN-β

Lymphocytes were treated with HBsAg or PLC/PRF/5 supernatants either during or after a 18 h incubation with IFN-β. In no instance cytotoxic assays, lymphocyte–K-562 conjugates, or reactions with MoAb were different from those observed on PLC/PRF/5 supernatants or HBsAg pre-treated lymphocytes not incubated with IFN-β.

HBV markers

None of the donors had HBsAg. Anti-HB_S antibodies were present in five donors, but the results obtained in these did not differ from those obtained in negative donors.

DISCUSSION

Culture supernatants from PLC/PRF/5 hepatocellular carcinoma contain a factor capable of suppressing NK cell activity of normal lymphocytes (Keong *et al.*, 1983). This cell line produces 22 nm HBsAg particles, which are phenotypically, morphologically and antigenically similar to those obtained from infected human sera (Alexander *et al.*, 1982). The purpose of the present study was to assess whether HBsAg derived from infected human sera directly mediated suppression of NK cell activity. A purified HBsAg preparation was used, since the PLC/PRF/5 cell line produces HBsAg, but not other HBV markers or the infectious virus (Alexander *et al.*, 1982). Our findings indicate that HBsAg (1) decreases *in vitro* NK activity, (2) interferes with the binding of effector lymphocytes to the target cells, (3) blocks FcR or structures closely related to them and (4) affects NK activity reversibly and in a dose-dependent manner, without being toxic to lymphocytes.

In previous studies we have shown that HBsAg can modify some immunological functions (Vierucci *et al.*, 1977, 1983, 1984). Phagocytosis of opsonized particles is affected, which requires the function of Fc and complement receptors. In addition, HBsAg inhibits PMN chemotaxis, which depends on microtubule and microfilament integrity. It is noteworthy that these cytoskeletal elements are important also in NK cell function, including the binding to target cells (Katz, Zaytoun & Lee, 1982).

HBsAg decreased lymphocyte–K-562 conjugates and the number of cells reactive with B73.1 MoAb. These modifications are specific, since HBsAg did not affect the binding of the other MoAb employed, including HNK-1, T8 and T11 MoAb, which recognize antigens present on subsets of B73.1(+) cells (Perussia *et al.*, 1983). In addition, HBsAg was not active upon K-562 cells. These cells bear FcR, but these receptors have different biochemical properties from the FcR on lymphocytes (Ichiki *et al.*, 1981). It is difficult to correlate a decrease in NK activity with blocking of FcR, since a role of FcR in NK function has not been defined (Herberman *et al.*, 1980). However, we speculate that HBsAg may react into receptor sites (possibly Fc receptor sites) on NK cell membrane. Experiments to verify this possibility (by means of Fc fragments) are in progress.

The ability of HBsAg to modulate NK cell membrane closely resembles the findings of Ali, Rees & Oxford (1984) on the effect of viral subunits on NK activity. They reported that haemoagglutinating subunits from influenza viruses are able to inhibit *in vitro* NK activity, and that haemoagglutinin binding to NK cell membrane is a prerequisite for inhibition to occur.

The effect of the presence of PMN in the assay system was tested, since PMN regulate NK cell activity (Kay & Smith, 1983) may be through the generation of metabolites of oxygen (Seaman *et al.*, 1983) which are produced by PMN in the presence of HBsAg (Vierucci *et al.*, 1977, 1983). However, no modification was observed, even though the PMN:lymphocyte ratio was varied widely to accord to the findings of Kay & Smith (1983).

IFN promotes the differentiation of pre-NK cells to mature cells, and augments the cytolytic activity of mature cells (Toy, 1983), but IFN-β did not protect NK cells from HBsAg-mediated inhibition, either before or during the contact with HBsAg. The same effect has been described in lymphocytes inhibited by haemoagglutinin from influenza viruses (Ali *et al.*, 1984).

Like influenza viruses (Ali *et al.*, 1984), HBV does not decrease *in vivo* NK activity of peripheral blood lymphocytes (Dienstag & Bhan, 1980; Dienstag *et al.*, 1982). This may indicate that some compensatory factor exists which *in vivo* prevents HBsAg from negatively influencing lymphocytes. On the other hand, it has been suggested that the level of NK cytotoxicity in any tissue is the resultant of a balance between positive and negative influences (Kay & Smith, 1983). For instance, peripheral blood lymphocytes of patients with rheumatoid arthritis exert normal NK cell activity, but this is reduced in synovial tissue lymphocytes (Dobloug *et al.*, 1982). Therefore, it is not inconceivable that NK cells may be inhibited in the liver by HBsAg on the hepatocellular membrane. Such a modification may be important in determining the outcome of HBsAg positive

chronic hepatitis and hepatocellular carcinoma, since NK cytotoxicity is probably involved in defence against viral infections and tumors (Biron & Welsh, 1982).

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