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Detection of cellular and humoral immunity to hepatitis B surface antigen (HBsAg) in asymptomatic HBsAg carriers

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SUMMARY

Cell mediated and humoral immunity to hepatitis B surface antigen (HBsAg) was studied in nine asymptomatic HBsAg carriers, nine patients with natural acquired immunity to HB infection and nine HB-susceptible donors. Peripheral T and B lymphocytes from all asymptomatic HBsAg carriers and all HB-immune donors studied were specifically induced into proliferation and anti-HBs secretion when stimulated with low doses of HBsAg (2–30 ng antigen protein/ml) *in vitro*. This activation was achieved by mixing purified B and/or T cells with optimal concentrations of autologous monocytic cells. T and B cells from the HB-susceptible donors were non-responsive under identical culture conditions. These data do neither substantiate the existence of a qualitative defect in T cell function, nor the absence of circulatory B cells capable of synthesizing anti-HBs *in vitro* in asymptomatic HBsAg carriers. Thus, the inability to mount a satisfactory antibody response to HBsAg *in vivo* might be a consequence of altered immune responsiveness to this antigen, which may be a relevant factor in the pathogenesis of asymptomatic HBsAg carriership.

Keywords hepatitis B surface antigen asymptomatic HBsAg carriers T- and B- cell activation

INTRODUCTION

The reason why asymptomatic hepatitis B surface antigen (HBsAg) carriers tolerate hepatitis B virus (HBV) is poorly understood. It has been suggested that the specific cell mediated response to HBsAg is impaired in asymptomatic HBsAg carriers, documented by the absence of stimulation and transformation of peripheral blood lymphocytes (PBL) by HBsAg (Laiwah, 1971; Warnatz, 1974; Tong *et al.*, 1975; Tiku *et al.*, 1978), inhibition of leucocyte migration (Laiwah, Chaudhuri & Anderson, 1973) and production of migratory inhibition factor (Gerber *et al.*, 1974). Furthermore, markedly impaired T cell function has been reported for asymptomatic HBsAg carriers (Levo *et al.*, 1981).

The absence of antibodies to HBsAg (anti-HBs) in HBsAg carriers has been regarded as evidence of B cell tolerance to HBV and recently a specific B cell defect in anti-HBs production has been suggested (Dusheiko *et al.*, 1983). Some authors, however, have reported the presence of immune complexes containing both HBsAg (Anh-Tuan & Novak, 1981; Palla *et al.*, 1983) and anti-HBs (Tiku, Beutner & Ogra, 1979) in serum from chronic HBsAg carriers. It seems, therefore, that

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HBsAg carriers are capable of mounting an antibody response to the outer coat of HBV, although inadequate for the development of protective immunity.

We have recently developed assay techniques for the evaluation of specifically induced cell-mediated and humoral immune responses to HBsAg in patients with naturally acquired immunity to HBV infection (Hellström, Sylvan & Lundbergh, 1985). The present study was undertaken to evaluate whether specific functional defects in the immune responsiveness of T and B lymphocyte populations from asymptomatic HBsAg carriers are demonstrable *in vitro* using antigen specific systems.

MATERIALS AND METHODS

Antigen. Purified HBsAg, subtype ad, was used (graciously provided by Dr M. Einarsson, Research Department, Biochemistry, AB Kabi, Stockholm, Sweden). It was purified by affinity chromatography from serum of asymptomatic HBsAg carriers as described (Einarsson, Kaplan & Utter, 1978; Einarsson, Kaplan & Pertoft, 1981). The HBsAg content was 25 μg antigen protein/ml.

Donor selection. Asymptomatic HBsAg carriers, HB immune and HB susceptible donors (nine in each group) were studied. The asymptomatic HBsAg carriers (five males, mean age 34 (range 22–39) years) lacked anti-HBs, had HBsAg titres varying between 1/10,000 and 1/100,000 (subtype ad in eight) and antibodies to hepatitis B core antigen (anti-HBc, 1/1,000–1/10,000). Eight exhibited stable titres (1/10–1/100) of antibodies to hepatitis e antigen (anti-HBe), while one donor fluctuated. Two carriers had undergone a percutaneous liver biopsy with normal histology and minimal inflammatory changes without evidence of hepatitis, respectively. Before being included in the study they were repeatedly tested over a 2–5 year period and their clinical status and liver function tests had been normal.

The HB-immune donors (five females, mean age 39 (range 24–49) years) had naturally acquired immunity to hepatitis B as judged by absence of HBsAg and presence of anti-HBs (1/1–1/5,000), anti-HBc (1/100–1/1,000). Six had a history of acute hepatitis (subtype ad in five and ay in one) and were bled 1–3 years after infection.

The HB-susceptible donors (seven females, mean age 34 (range 22–55) years) lacked HBsAg, anti-HBs and anti-HBc and were randomly selected individuals without known previous exposure to HBV.

At the time of bleeding all donors from the three groups were considered healthy by clinical and laboratory examination (ESR, blood and urine analysis and liver function tests). Hepatitis B serology was analysed by commercially available kits (AUSRIA II, AUSAB, CORAB and anti-HBe Diagnostic, Abbott Laboratories, North Chicago, Illinois, USA).

Preparation of cells. PBL was highly purified from heparinized blood by gelatin-sedimentation, iron-treatment and Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden) gradient centrifugation (Perlmann *et al.*, 1976). T-cells were isolated by rosetting PBL with neuraminidase treated sheep erythrocytes (SRBC) and recovered after osmotic lysis of SRBC (Lehtinen *et al.*, 1980). B-cells were recovered from the non-rosetting fraction after a second rosetting procedure and contained 73% (range 63–82%) B-cells as assessed by reactivity with the monoclonal antibody B1 (Stashenko *et al.*, 1980) purchased from Coulter Electronics Ltd; Luton, Bedfordshire, UK. Monocytic cells (Mϕ) were isolated by gelatin sedimentation, bovine albumin gradient centrifugation and adherence (Hellström *et al.*, 1985). The purity of T, B and Mϕ fractions were similar for the three donor groups.

Proliferation assay. T-cells (10^5/well) and autologous Mϕ (10^4/well) were resuspended in Hepes buffered RPMI 1640 (Biocult Laboratories, Paisley, Scotland) supplemented with antibiotics, glutamine and 10% heat inactivated normal AB^+ human serum (negative for HBV markers) and cultured with HBsAg (2 ng–2·5 μg/ml) for 5–8 days at 37°C. The cells were pulsed with ^{3}H-thymidine during the last 16–18 h of incubation (Hellström *et al.*, 1985). Data are given as ct min (mean ct min from triplicate or quadriplicate) of antigen stimulated cultures corrected for ct min values in T-Mϕ control cultures without antigen.

B-cell activation assay. B-cells ($2{\cdot}5 \times 10^4$/well) and autologous Mϕ ($2{\cdot}5 \times 10^3$/well) were cultured

with autologous T-cells (T/B ratio 0·25–8·0) and HBsAg (0·25 ng–2·5 μg/ml) for 4 days in medium supplemented with 20% foetal bovine serum (FBS, GIBCO, Grand Island, New York, USA) (2–4 wells/culture). On the fourth day medium was changed to contain 5% FBS and the cell cultures incubated for additional 6–8 days. For screening procedures T and B cells were cultured with 2·5 ng antigen protein/ml for $7\frac{1}{2}$ days in medium containing 20% FBS. Culture supernatants were analysed for total IgG and IgM content by ELISA (Hellström *et al.*, 1985). For detection of anti-HBs, microtitre plates coated with HBsAg subtype ad (150 ng/ml, graciously provided by Dr M. Kuhns, Diagnostic Division, Abbott Laboratory, North Chicago, Illinois, USA) were used. Culture supernatants were diluted 2×10^{-1} in phosphate buffered saline (PBS)-Tween and incubated overnight at 20°C. Alkaline phosphatase conjugated (Engvall & Perlmann, 1972) rabbit anti-human γ and μ chains (Dakopatts A/S, Copenhagen, Denmark) were diluted 10^{-3} in PBS-Tween-4% FBS, pooled, and incubated overnight at 20°C. Linear standard curves were generated for each experiment by serial two-step dilutions of anti-HBs standard (Hoechst, Behringwerke AG, Diagnostics, Marburg, Germany) in PBS-Tween-4% FBS (0·5–40 miu/ml). The absorbance at 405 nm was measured after 1–2 h of incubation with substrate. Wells incubated with PBS-Tween-4% FBS, conjugate and substrate was used to blank the spectrophotometer before reading the plate. Furthermore anti-HBs was assayed by the commercially available Enzygnost Anti-HBs (Hoechst) and AUSAB-EIA (Abbott). Anti-HBs standards and culture supernatants were diluted in anti-HBs negative control human serum to 1–133 miu/ml and 2×10^{-1}, respectively. Incubations were performed as above. The spectrophotometer was blanked with wells incubated with control human serum, conjugate and substrate. Data are given as ng or miu/ml/10^5 HBsAg stimulated B cells corrected for background activation in control cultures without antigen.

Statistics. The levels of significance (P values) were obtained using either Student's t-test or Student's impaired t-test when appropriate.

RESULTS

HBsAg induced T cell proliferation

Significant proliferative responses were registered in antigen stimulated cultures from asymptomatic HBsAg carriers and HB-immune donors but not in corresponding cultures from HB-susceptible controls. The HBsAg induced ^{3}H-thymidine incorporation was of short duration and observed at different days (varying between 5 and 8 days after initiation of culture) for individual donors. Maximal specific stimulation was obtained with 2–30 ng antigen protein/ml for all HBsAg carriers and HB immune donors tested. This response was specifically induced by HBsAg since it was not obtained in corresponding control cultures from HB susceptible donors (Fig. 1). Suppression of the specific T cell response was noted at HBsAg concentrations ranging between 30 and < 250 ng/ml.

In contrast high concentrations of HBsAg (250 ng–2·5 μg/ml) induced an increase in ^{3}H-thymidine incorporation for some asymptomatic HBsAg carriers and HB-immune donors. This response, however, was of non-specific nature, since it was also obtained in cell cultures from HB-susceptible donors (data not shown).

The value of maximal stimulation obtained for each individual was considered for statistical analysis and compiled in Fig. 2. Both HBsAg carriers and HB-immune donors exhibited proliferative responses induced by HBsAg significantly different from both unstimulated cultures from the same individual ($P < 0{\cdot}01$) and from antigen-stimulated control cultures from susceptible donors ($P < 0{\cdot}001$). The ^{3}H-thymidine incorporation in cultures from the nine asymptomatic HBsAg carriers (8747 ct min) was higher compared to HB-immune donors (6015 ct min). The difference between the two groups, however, did not reach statistical significance.

HBsAg induced anti-HBs secretion

Significant anti-HBs secretion was observed in HBsAg induced B cell culture supernatants from asymptomatic HBsAg carriers and HB-immune donors but not from corresponding cultures from HB-susceptible controls. The antigen induced B cell activation was T cell dependent and registered at optimal proportions between autologous T and B cells, varying for individual donors (being 0·5–

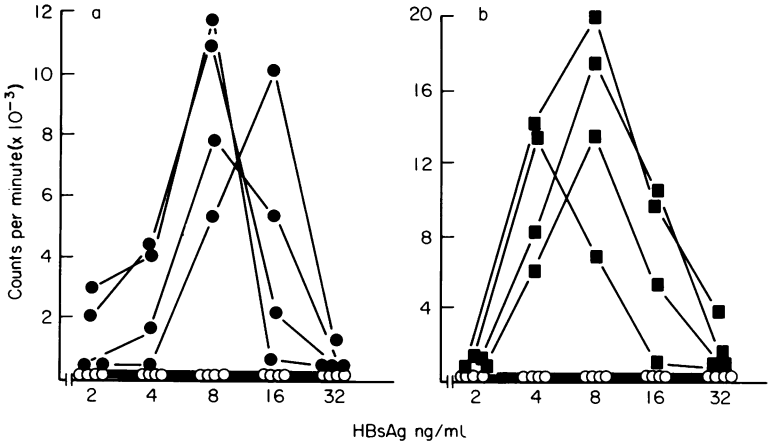

Fig. 1. Antigen dose-dependency of HBsAg induced T cell proliferation. 1×10^5 peripheral T cells from (a) four HB-immune (●) or (b) four asymptomatic HBsAg carriers (■) and four HB-susceptible (○) donors were co-cultured with 1×10^4 autologous Mϕ in the presence of different concentrations (2, 4, 8, 16 and 32 ng/ml) of HBsAg for 5–8 days. Proliferation was measured by ^{3}H-thymidine incorporation. Each value depicted is corrected for background incorporation in T-Mϕ cultures without antigen.

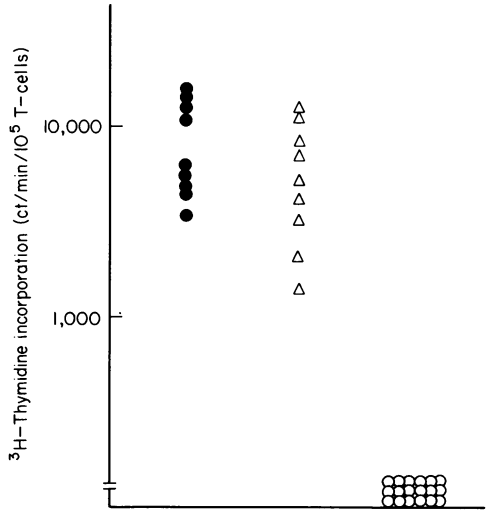

Fig. 2. HBsAg induced T lymphocyte proliferation in asymptomatic HBsAg carriers and HB-immune donors. Maximal ^{3}H-thymidine incorporation in HBsAg stimulated cultures from nine asymptomatic HBsAg carriers (●), nine HB-immune donors (△) and corresponding 18 control cultures from nine HB-susceptible donors (○). 1×10^5 T lymphocytes were supplemented with 10% autologous Mϕ and cultured with optimal concentrations of HBsAg (2–30 ng antigen protein/ml) for 5–8 days. Each value depicted is corrected for background incorporation in lymphocyte cultures without antigen.

8·0 for asymptomatic HBsAg carriers and 0·25–2·0 for HB-immune donors). Maximal anti-HBs secretion was obtained with 2·5–25 ng antigen protein/ml for all HBsAg carriers and HB-immune donors tested. This secretion was specifically induced since it was not obtained in corresponding control cultures from HB-susceptible donors (Fig. 3). Moreover high HBsAg concentrations (250 ng–2·5 μg/ml) were suppressive for the induction of anti-HBs secretion (data not shown).

The titres of secreted anti-HBs ranged between 10 and 160 miu/ml and were similar for both donor groups (carriers and immune) (Fig. 4a).

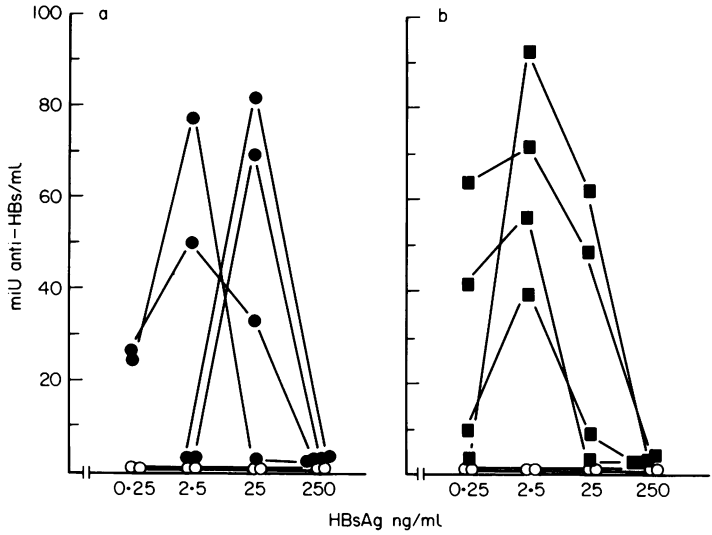

Fig. 3. Antigen-dose dependency of HBsAg induced anti-HBs secretion. Peripheral B-cells from (a) four HB-immune (●) or (b) four asymptomatic HBsAg carriers (■) and two HB-susceptible donors (○) were cocultured with autologous T-cells at T/B ratios (a) 0·5 and (b) 0·5 or 4·0 in the presence of different concentrations (0·25, 2·5, 25 and 250 ng/ml) of HBsAg for 10–12 days. Anti-HBs secretion are given as m iu anti-HBs/ml/10^5 B-cells corrected for background activation in control cultures without antigen.

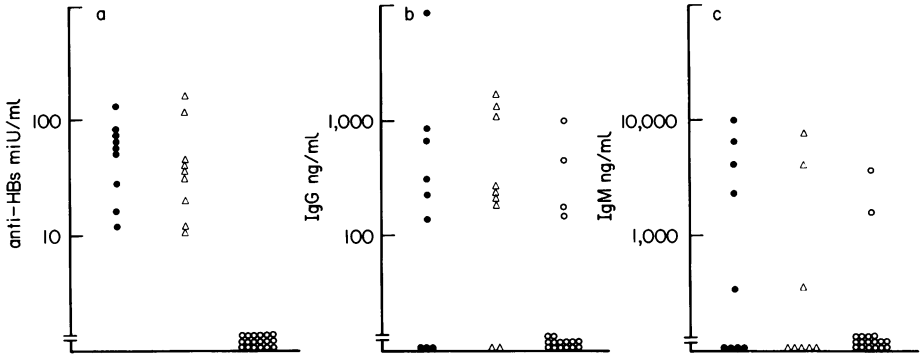

Fig. 4. HBsAg induced anti-HBs secretion in asymptomatic HBsAg carriers and HB-immune. Anti-HBs (a), total IgG (b) and total IgM (c) secretion/10^5 B cells in HBsAg stimulated cultures from nine asymptomatic HBsAg carriers (●), nine HB-immune donors (△) and corresponding 18 control cultures from nine HB-susceptible donors (○). B-cells were cocultured with autologous T-cells at optimal T/B ratios (varying between 0·25 and 8·0 for individual donors) and HBsAg (2·5 ng/ml) for $7\frac{1}{2}$ days. Each value depicted is corrected for background activation in cell cultures without antigen.

IgG and IgM antibodies were also quantitated in the culture supernatants (Figs 4b, c). HBsAg induced IgG secretion was demonstrated for six asymptomatic HBsAg carriers and seven HB-immune donors, whereas culture supernatants from three carriers and two HB-immune donors contained only IgM. B cells from two carriers and one immune secreted detectable amounts of both immunoglobulin classes. No specific anti-HBs secretion was observed for the HB-susceptible donors, although IgG and/or IgM was registered for four of the nine donors.

Both HBsAg carriers and HB-immune donors exhibited anti-HBs secretion induced by HBsAg, significantly different from both unstimulated cultures from the same individual ($P < 0{\cdot}05$) and from antigen-stimulated control cultures from susceptible donors ($P < 0{\cdot}01$).

DISCUSSION

In the present study we demonstrated that peripheral T and B lymphocytes from asymptomatic HBsAg carriers are inducible into proliferation and anti-HBs secretion by HBsAg *in vitro*. Maximal proliferative responses and the amount of anti-HBs measured in culture supernatants after HBsAg stimulation was of the same magnitude as for donors with naturally acquired immunity to HB.

The activation of sensitized peripheral T and B lymphocytes by soluble antigens *in vitro* is strictly regulated by a number of factors, e.g. the proportion of accessory and regulatory T cells, factors produced by these cells and the concentration of the stimulating antigen used (Fauci, 1982). Recently we reported (Hellström *et al.*, 1985), monocyte requirements for the induction of HBsAg induced proliferation in highly purified PBL from donors with naturally acquired immunity to HB. Monocytic cells were shown to be a prerequisite for the induction of HBsAg induced lymphocyte transformation, while excessive amounts effectively suppressed humoral as well as cell-mediated responses to HBsAg. Similarly, addition of supraoptimal numbers of autologous monocytes were suppressive for the HBsAg induced T lymphocyte proliferation in asymptomatic HBsAg carriers (data not shown). Thus, it is conceivable that the utilization of crude lymphocyte preparations with uncontrolled Mϕ contamination is one reason for the absence of lymphocyte transformation reported by other groups evaluating lymphocyte blastogenesis to HBsAg in chronic HBsAg carriers *in vitro* (Laiwah, 1971; Warnatz, 1974, Tong *et al.*, 1975; Tiku *et al.*, 1978; Hanson *et al.*, 1984).

Moreover, compared to previous reports of HBsAg induced lymphocyte activation the current study employed extremely low concentrations of HBsAg for the induction of specific T cell proliferation and anti-HBs secretion (2–30 ng antigen protein/ml) in both asymptomatic HBsAg carriers and HB immune donors. Increasing concentrations of HBsAg beyond the optimal requirements were suppressive. Identical results were furthermore obtained with the commercially available Hepatitis B vaccine (Merck Sharp & Dohme Research Laboratories) (P.E.S. Sylvan & U.B. Hellström, unpublished data).

In contrast, *in vivo* concentrations of HBsAg in asymptomatic HBsAg carriers (e.g. 2·5 μg/ml) could for some asymptomatic HBsAg carriers and HB immune donors induce proliferative responses, however, of a non-specific nature, since it was also registered in cell cultures from HB susceptible donors. These responses were 2–4-fold higher compared to the specifically induced ^{3}H-thymidine incorporations at low antigen concentrations.

HBsAg induced anti-HBs secretion *in vitro* has not previously been reported for asymptomatic HBsAg carriers. Dusheiko *et al.* (1983) reported anti-HBc but no anti-HBs production in a PWM driven B cell activation system using PBL from chronic HBsAg carriers. Discrepancies between their data and ours indicate, that culture conditions used for PWM induced polyclonal B cell activation of PBL are inappropriate for achieving specifically induced anti-HBs secretion in chronic HBsAg carriers.

Although no quantitative difference was registered in the amount of anti-HBs production in asymptomatic HBsAg carriers compared to HB immune donors, the quality of the specific antibodies produced by carriers might still be altered. Whether asymptomatic HBsAg carriers in fact do produce anti-HBs of different immunoglobulin classes or subclasses *in vivo* is not fully elucidated. If produced, these antibodies could combine with circulatory HBsAg in excess and form immune complexes. Both HBsAg and anti-HBs containing immune complexes have indeed been demonstrated in some chronic HBsAg carriers (Anh-Tuan & Novak, 1981; Palla *et al.*, 1983; Tiku, Beutner & Ogra, 1979). It is conceivable that such immune complexes could down-regulate the specific B-cell activation, either directly or through the regulatory capacities exerted by subsets within the T-cell compartment (Uhr & Möller, 1968; Setcavage & Kim, 1980). Such immune complexes may also interfere with the production of anti-idiotypic antibodies important for the B-cell response to HBsAg (Kennedy & Dreesman, 1984).

Moreover, since high concentrations of HBsAg were suppressive for specific B cell activation *in vitro*, it is possible that high *in vivo* concentrations of HBsAg in serum from asymptomatic HbsAg carriers could exert immunoregulatory effects of a suppressive nature.

Thus, our data do neither substantiate the existence of a defect T-cell function, nor the absence

of circulatory B cells capable of synthesizing anti-HBs *in vitro* in patients with asymptomatic HBsAg carriership. Instead we conclude that the inability to mount a satisfactory antibody response to HBsAg *in vivo* may be an effect of altered immune function on the response to the outer coat of HBV, limiting both the humoral immune response to HBsAg and the effector lymphocyte response to HBsAg containing hepatocytes.

Studies of immunoregulatory cell-phenotypes with the aid of T4 and T8 monoclonal antibodies, identifying the T-inducer and T-suppressor/cytotoxic cell population, respectively, have reported divergent findings. In some studies a profound shift in balance favouring the $T8^+$ T cell population in asymptomatic HBsAg carriers was demonstrated (Dienstag, 1984).

So far we do not know the phenotype or effector cell function of the HBsAg sensitized T cell population(s) in asymptomatic HBsAg carriers and HB-immune donors, nor whether differences regarding these properties exists between the two groups. These queries can now be addressed. By using our assays in conjunction further dissection will be possible of the immunoregulatory network in patients with normal and altered immunresponses to HBsAg.

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