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Cellular immunity to the hepatitis B virion in acute hepatitis type B

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SUMMARY

Ten patients were studied serially during acute hepatitis type B for lymphocyte sensitization to the hepatitis B surface antigen (HBsAg) and to the complete hepatitis B virion (Dane particle). Using the lymphocyte transformation test, sensitization to purified HBsAg was not observed during the first 10 days of illness but became detectable later, being particularly evident during convalescence, while sensitization to antigens of the complete virion, other than HBsAg, was demonstrable as soon as at the onset of symptoms, often at the time of maximum liver cell damage. These results indicate that in the course of acute hepatitis type B, lymphocyte sensitization to other antigens of the complete virion precedes that to HBsAg and may be of greater pathogenetic importance.

Keywords acute hepatitis type B lymphocyte transformation test hepatitis B surface antigen Dane particles cellular immunity

INTRODUCTION

Cell-mediated immunity to viral antigens is thought to play an important role in hepatitis B virus (HBV) infection and in the past decade several studies have been conducted to investigate during acute and chronic infection lymphocyte response to the hepatitis B surface antigen (HBsAg), considered the most likely target antigen on the surface of infected hepatocytes (Alberti *et al.*, 1976). Only a few authors, however, have used purified HBsAg (De Gast, Houwen & Nieweg, 1973; Ibrahim, Vyas & Perkins, 1975), while in the majority of *in vitro* experiments the lymphocyte response was assessed using high titre HBsAg positive serum or partially purified HBsAg (Yeung Laiwah, 1971; Dudley, Giustino & Sherlock, 1972b; Reed *et al.*, 1974; Lee *et al.*, 1977) that in both instances may have contained other components of the complete HBV particle, the role of which in stimulating the cellular response has not been evaluated. Recently Dienstag & Bhan (1980) and Chisari *et a.* (1981) have failed to detect lymphocyte cytotoxicity to hepatoma cells that express HBsAg on their membrane but do not support virus replication during HBV infection.

These observations and the association found between active virus replication and liver damage (Realdi *et al.*, 1980; Hoofnagle *et al.*, 1981) would suggest that other antigens of the complete virion, besides HBsAg, may be relevant in eliciting cytolytic effector mechanisms when expressed on the surface membrane of infected cells.

In the present stuy we have assessed lymphocyte sensitization to antigenic components of the

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complete hepatitis B virus particle, namely the Dane particle (DP), during acute infection and have compared it with the lymphocyte response obtained in the presence of purified HBsAg particles free of complete virions.

MATERIALS AND METHODS

Patients. Ten patients (nine male and one female; mean age 24·60 ± 10·46 years) with acute hepatitis type B were included in this study. Acute hepatitis B was diagnosed on the basis of a recent onset of hepatitis symptoms and jaundice, associated with a sharp rise in alanineaminotransferase (ALT) levels (mean peak ALT value ± s.d. = 2,959 ± 1,499 iu/l). All patients were first studied for lymphocyte sensitization to HBsAg and to DP within 10 days after clinical onset, when HBsAg and hepatitis B e antigen (HBeAg) were both positive in serum, and then followed serially during acute phase and convalescence. All 10 patients showed complete recovery from the illness, with normalization of transaminases and clearance of HBsAg from serum.

Fifteen sex and age matched healthy subjects, with no evidence of previous exposure to hepatitis B virus (negative by radioimmunoassay for HBsAg and for antibody to HBsAg [anti-HBs] and to hepatitis B core antigen [anti-HBc]) served as controls. At least one of these control cases was included in each batch of assays.

Preparation of purified HBsAg and of DP. Purified HBsAg was prepared by affinity chromatography on Sepharose 4 B coupled with monoclonal anti-HBs (a kind gift by Dr H.C. Thomas, Royal Free Hospital, London) starting from a serum of a healthy HBsAg carrier, who was positive in serum for antibody to HBeAg (anti-HBe) and negative for HBV-DNA polymerase and was proved free of hepatitis B core antigen (HBcAg) in the liver by direct immunofluorescence. The final HBsAg preparation was adjusted at 150 μg/ml in normal saline, with HBsAg titre of 1 : 1,000 by reverse passive haemagglutination (RPHA). This preparation was negative by immunodiffusion against anti-human plasma protein sera and was proved to contain only 22 nm particles when analysed by electron microscopy.

DP enriched pellets were prepared by density gradient ultracentrifugation (Robinson & Greenman, 1974) from the serum of an asymptomatic HBsAg carrier positive for HBeAg and with high HBV-DNA polymerase level in serum. After dialysis against normal saline the DP preparation was adjusted at a protein concentration of 800 μg/ml with HBsAg titre of 1 : 8,000 by RPHA. This preparation was highly positive for HBV-DNA polymerase activity (specific ^{3}H-TTP incorporation = 6,600 ct/min/50 μl) and was found to contain many intact DPs, together with scarce 22 nm and tubular HBsAg forms when studied by electron microscopy. The HBsAg and DP preparations were kept frozen at −30°C in small aliquots to be used for *in vitro* assays throughout the period of investigation. Before each batch of experiments HBsAg titre was equilibrated in the two antigenic preparations (see below).

Lymphocyte transformation. Twenty millilitres of heparinized venous blood were obtained from each subject and peripheral blood mononuclear cells (PBMc) were prepared by Ficoll-Hypaque density gradient separation (Böyum, 1968); cells were then washed twice and resuspended at 2×10^6/ml in RPMI 1640, enriched with 10% fetal calf serum, 4 mM glutamine, 100 u/ml penicillin, 100 μg/ml streptomycin and 2 μg/ml amphotericin B. Two hundred thousand PBMc in 0·2 ml of RPMI 1640 tissue culture medium were incubated in plastic microplates (Falcon Plastics) either alone (unstimulated culture) or with purified HBsAg or DP (stimulated culture). Before using in the experiments, HBsAg and DP were equilibrated for HBsAg titre (1 : 1,000 by RPHA) and were then added to lymphocytes at four serial dilutions (1 : 15, 1 : 30, 1 : 300, 1 : 3,000) with a final protein concentration of 10 μg/ml, 5 μg/ml, 0·5 μg/ml and 0·05 μg/ml for HBsAg and of 6·5 μg/ml, 3·25 μg/ml, 0·325 μg/ml and 0·0325 μg/ml for DP, respectively. All experiments were performed in triplicate. Cultures were incubated for 6 days at 37°C in 95% air and 5% Co_2. Six hours before harvesting, 1 μCi of ^{3}H-thymidine (specific activity 1 Ci/mM, Sorin) was added to each well. Lymphocytes were collected onto glass discs using an automatic cell harvester (Skatron, Lierbyen, Norway). Radioactivity in the filters, was measured in a liquid scintillation counter (Packard). Results were expressed as stimulation index (SI), calculated in this manner:

$$SI = \frac{^3H \text{ ct/min stimulated culture}}{^3H \text{ ct/min unstimulated culture}}.$$

Statistical analysis was done by the Student's *t*-test.

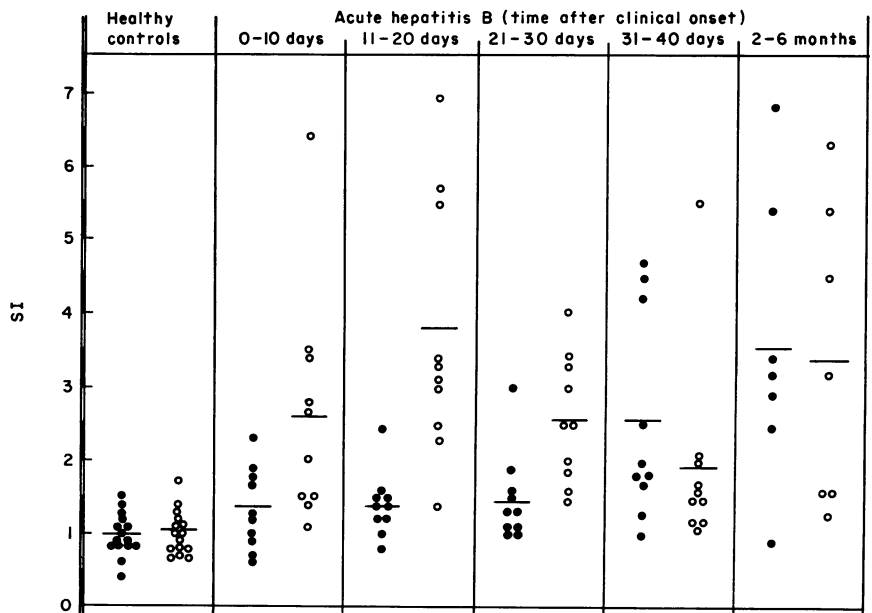
Serum HBV markers. Serum samples were obtained weekly from the 10 acute hepatitis B cases to be tested for HBsAg, HBeAg and anti-HBe by commercially available RIA kits (Abbott Laboratories). HBsAg titres in patients sera and in purified preparations were determined by reverse passive haemagglutination (RPHA-Hepatest, Wellcome). HBV-DNA polymerase levels were measured by the method described by Alberti, Pontisso & Realdi (1981).

Electron microscopy. Electron microscopy of HBsAg and DP preparations was carried out using negative staining with 3% tungsto-phosphoric acid pH 6·0 as previously described (Alberti *et al.*, 1978a).

RESULTS

Lymphocyte stimulation with purified HBsAg and with DP in acute hepatitis B

Purified HBsAg and DP were equilibrated to the same HBsAg titre (1:1,000 by RPHA) and then tested at four different dilutions against patients' lymphocytes as well as control lymphocytes. The value of maximum SI obtained in each case was considered for statistical analysis. In the 15 seronegative healthy control subjects mean SI with HBsAg and with DP was 0·97±0·07 (mean±s.e.) and 1·05±0·08, respectively. The results obtained in the 10 patients with acute hepatitis B tested serially during acute phase and after recovery are shown in Fig. 1. Mean SI with



	Healthy controls	0-10 days	11-20 days	21-30 days	31-40 days	2-6 months
Mean SI with HBsAg (±s.e.)	0·97±0·07	1·34±0·17	1·40±0·13	1·48±0·19	2·55±0·43	3·57±0·73
P vs controls	—	n.s.	<0·05	<0·05	<0·01	<0·005
Mean SI with DP (±s.e.)	1·05±0·08	2·63±0·49	3·81±0·53	2·57±0·26	1·94±0·40	3·41±0·76
P vs controls	—	<0·005	<0·001	<0·001	<0·05	<0·01
Mean SI with HBs Ag *vs* mean SI with DP	n.s.	*P*<0·05	*P*<0·001	*P*<0·005	n.s.	n.s.

Fig. 1. SI in the lymphocyte transformation test with purified HBsAg (●) and with Dane particles (DP) (○) in controls and in acute hepatitis B cases tested serially during acute illness and after recovery.

purified HBsAg did not differ compared to that in healthy controls during the first 10 days of acute hepatitis B, while it was slightly but significantly increased from 10 to 30 days after onset and further raised during convalescence. In contrast, mean SI with DP was significantly increased compared to that in controls both during acute phase and convalescence. During the first 10 days of illness patients were found to respond to DP but not to HBsAg, while from 10 to 30 days after onset, although patients were sensitized to both preparations, mean SI with DP were significantly higher than those with HBsAg. In contrast no differences in SI with DP and with HBsAg were recorded during convalescence when patients appeared equally sensitized to both preparations.

When the temporal behaviour of the lymphocyte transformation test with HBsAg and with DP was analysed in each of the 10 acute hepatitis B cases in relation to transaminase levels and to HBV markers, in most cases sensitization to DP had already become detectable during the early phase, at the time of peak transaminase levels and before clearance of HBeAg, while sensitization to HBsAg was demonstrable later in the course of illness, after substantial reduction in HBsAg titre and in transaminase levels. At this time most patients appeared sensitized to both antigen preparations, thus precluding evaluation of the role of antigens other than HBsAg in eliciting the lymphocyte response to DPs. An example of these changes in one of the 10 patients studied is reported in Table 1.

Table 1. Cell-mediated immunity to HBsAg and to DP in a patient with acute hepatitis B tested serially during acute phase and after recovery

				SI	
Time after clinical onset	HBsAg/anti-HBs	HBeAg/anti-HBe	ALT*	HBsAg	DP
0–10 days	512†/neg.	+/neg.	2,600	0·9	3·5
11–20 days	4096/neg.	+/neg.	2,200	0·7	3·4
21–30 days	512/neg.	neg./neg.	470	1·4	1·9
31–40 days	128/neg.	neg./+	430	1·8	3·3
4 months	neg./neg.	neg./+	18	5·4	3·0

* ALT = serum alanine aminotransferase (normal value < 50 iu/l).
† HBsAg titre by reverse passive haemagglutination (RPHA).

DISCUSSION

The hepatitis B virus is not thought to be directly cytopathic. Liver damage in acute and chronic infection may depend on cytotoxic lymphocytes reacting with viral antigens on the surface of infected hepatocytes (Dudley, Fox & Sherlock, 1972a; Edgington & Chisari, 1975). The target cell surface antigen remains undefined, but there is evidence suggesting that its expression in the liver may depend on active virus replication, in view of the close association between complete virus production and liver cell damage (Realdi *et al.*, 1980; Hoofnagle *et al.*, 1981). Recent evidence indicates that HBsAg is not a target of effector mechanisms if expressed on cells containing integrated HBV genomes but not supporting virus replication (Dienstag & Bhan, 1980; Chisari *et al.*, 1981).

In the present study we have explored the possibility that lymphocytes from patients with acute, HBV related, liver damage could be sensitized to antigenic components of the virion, other than HBsAg. Lymphocytes from these cases were therefore cultured in the presence of DPs. Since these particles express HBsAg on their surface, lymphocyte sensitization to an equivalent amount of HBsAg, free of other virion components, was also studied in parallel experiments. Early in the course of acute hepatitis B, lymphocytes were sensitized to the DP preparation but not to HBsAg, indicating involvement of other antigens of the complete virion. In most cases this type of immune

response was already detectable a few days after clinical onset, at the time of maximum liver cell damage and before clearance of HBeAg. In contrast, sensitization to HBsAg became detectable later in the course of the illness, after the peak of transaminase levels and, in agreement with previous studies conducted with purified HBsAg (Erard, 1974; Tong *et al.*, 1975; Desaules *et al.*, 1976), was maximum during convalescence. At this time patients' lymphocytes proliferated with both antigenic preparations, making it impossible to further discriminate between HBsAg and other virion components in the response to the DP preparation. These results support the hypothesis that cell-mediated reactions to antigens of the complete HBV particle, other than HBsAg, may be of greater pathogenetic importance in causing the lysis of infected hepatocytes during acute hepatitis type B. The antigenic components of the DP involved in this reaction remain undefined. They may be represented by additional antigenic sites, distinct from HBsAg, that have been suggested to exist on DPs (Neurath *et al.*, 1976; Neurath, Strick & Huang, 1980; Alberti *et al.*, 1978b). However, a role of internal virion components cannot be excluded, since processing of virus particles by macrophages during the *in vitro* culture could certainly expose them to lymphocytes. Among these antigenic determinants, the hepatitis B core antigen (HBcAg) appears as a likely candidate to represent a target for sensitized lymphocytes. Indeed HBcAg has been demonstrated on the surface of infected hepatocyes (Trevisan *et al.*, 1982) and cytotoxic T lymphocytes with specificity for HBcAg on liver cell surface have been recently detected in autologous cytotoxicity experiments (Mondelli *et al.*, 1982). However both these observations were made in chronic HBV infection, where immunopathogenetic mechanisms may differ from those involved in acute, HBV related, liver damage.

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