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Cell-mediated immunity to hepatitis B surface antigen in man

R. G. HANSON, J. H. HOOFNAGLE, G. Y. MINUK,* R. H. PURCELL & J. L. GERIN *Liver Diseases Section, Digestive Diseases Branch, National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases; Hepatitis Section, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda and Molecular Virology and Immunology Division, Georgetown University, Rockville, Maryland, USA*

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SUMMARY

An aqueous preparation of hepatitis B virus (HBV) vaccine was used as an intradermal skin test antigen to assess delayed hypersensitivity to hepatitis B surface antigen (HBsAg). Thirty-five persons were tested including 10 individuals seronegative for all HBV markers, 10 positive for HBsAg (chronic carriers) and 15 positive for antibody to HBsAg (anti-HBs), five of whom had received the HBV vaccine. All patients were also studied for lymphocyte blastogenic responses to phytohaemagglutinin, concanavalin A, pokeweed mitogen and purified HBsAg. Only one individual had a positive delayed skin test reaction to HBsAg. This person had received the HBV vaccine and had high titres of anti-HBs in serum. However, neither this individual nor any other subject exhibited a positive lymphocyte blastogenic response to HBsAg *in vitro*. Thus, delayed hypersensitivity skin test reactivity to HBsAg was not detected after natural infection with HBV and was rarely present in hyperimmunized individuals. *In vitro* assays of immune responsiveness failed to demonstrate cellular immunity to HBsAg even in hyperimmunized persons. These studies provide no evidence that cell-mediated immunity to HBsAg plays a role in the immunopathogenesis of acute or chronic type B hepatitis.

Keywords hepatitis B surface antigen chronic hepatitis hepatitis B virus vaccine delayed hypersensitivity

INTRODUCTION

The role of the immune system in the pathogenesis of acute and chronic type B hepatitis is not well understood. The immunological phenomena that lead to the clearance of hepatitis B virus (HBV) and hepatitis B surface antigen (HBsAg) in acute type B hepatitis and that are apparently deficient in chronic type B hepatitis have not been thoroughly elucidated. It has been suggested that delayed hypersensitivity or the cellular immune response to HBsAg plays an important part in the clearance of HBV (Dudley, Fox & Sherlock, 1972a, Dudley, Giustino & Sherlock, 1972b). Studies of the cellular immune response to HBsAg in acute and chronic type B hepatitis have been interpreted as supporting this hypothesis (De Gast, Houwen & Nieweg, 1973; Gerber, Phuangsab & Vittah, 1974; Irwin *et al.*, 1974; Reed *et al.*, 1974, De Moura, Vernace & Paronetto, 1975; Ibrahim, Vyas & Perkins, 1975; Tong *et al.*, 1975; Aldershvile *et al.*, 1977; Lee *et al.*, 1977; Beutner, Tiku & Ogra, 1978; Tiku *et al.*, 1978). The recent availability of the HBV vaccine (Purcell & Gerin, 1978), which consists of purified HBsAg that is safe for human use, has allowed us to reassess delayed

*Present address: Department of Gastroenterology, University of Calgary, Calgary, Alberta, Canada.
Correspondence: Dr Jay H. Hoofnagle, Liver Diseases Section, NIADDK, Bldg 10, Room 4-D-52, NIH, Bethesda, Maryland 20205, USA.

hypersensitivity to this viral antigen by means of intradermal skin testing and *in vitro* assays of cellular immune responsiveness.

MATERIALS AND METHODS

Serological assays. Hepatitis B surface antigen (HBsAg) and antibody (anti-HBs) and antibody to hepatitis B core antigen (anti-HBc) were assayed by solid phase radioimmunoassays (Ausria-II, Ausab and Corab: Abbott Laboratories, North Chicago, Illinois, USA) and the amount or titre of each were quantified by end-point titration. Subtyping of HBsAg and anti-HBs were performed by immunodiffusion and radioimmunoassay (Hoofnagle *et al.*, 1977). Hepatitis B e antigen (HBeAg) and antibody (anti-HBe) were measured by immunodiffusion.

Patients. Thirty-five patients entered into this study. These included 10 patients with chronic type B hepatitis (chronic HBsAg carriers), 10 subjects who had recovered from acute type B hepatitis and were seropositive for anti-HBc and/or anti-HBs (immune individuals), five persons who had received the HBV vaccine and were seropositive for anti-HBs (vaccine recipients), and 10 healthy persons who were seronegative for all HBV markers (susceptible individuals). The 10 HBsAg carriers all had HBsAg and HBeAg in serum for at least 1 year and had chronic hepatitis as determined by liver biopsy (seven with chronic active hepatitis and three with chronic persistent hepatitis). The ranges of serum alanine and aspartate aminotransferase activities (ALT and AST) in this groups were: ALT 25–1,075 (mean 208) u/l, and AST 28–505 (mean 108) u/l. Serum bilirubin levels were normal. None of these patients was receiving corticosteroid or other immunosuppressive therapy. The other subjects (immune, vaccinated and susceptible individuals) were healthy and all had normal serum aminotransferase levels (ALT < 44 u/l; AST < 31 u/l) at the time of serological evaluation and skin testing. All subjects gave written, informed consent for participation and all details of this study were approved by the clinical research subpanel of the National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases of the National Institutes of Health.

Protocol. Subjects initially had blood drawn for determination of HBV markers, for routine serum biochemical tests and for assays of lymphocyte blastogenesis. They then received five intradermal skin tests. Skin test antigens were given in 0·1 ml amounts and were read for erythema and induration at 4, 24 and 48 h. A reaction with both erythema and induration measuring more than 5 mm in diameter at 24–48 h was considered positive. The skin test antigens used were: trichophyton (Dermatophyton, dilution 1:30; Hollister–Stier, Spokane, Washington, USA), candida (Dermatophyton 'O': dilution 1:100, Hollister–Stier), mumps (Eli Lilly and Company, Indianapolis, Indiana, USA), tuberculin purified protein derivative (PPD) (Tubersol, Connaught Laboratories Limited, Willowdale, Ontario, Canada) and HBsAg (4 μg). The HBsAg preparation used was a specially prepared formulation of HBV vaccine (Lot A8, subtype adw) which had been produced and evaluated by the National Institute of Allergy and Infectious Diseases (Purcell & Gerin, 1978). This lot of HBV vaccine had been thoroughly tested for purity, safety and immunogenicity, and its use as a skin test antigen was approved by the Bureau of Biologics, Food and Drug Administration. The final product was in aqueous solution and contained neither albumin, adjuvant nor detectable formalin. The concentration of HBsAg was 40 μg/ml. Selected individuals were retested using multiple concentrations of the HBsAg 0·1, 0·4, 1, 4 and 20 μg in 0·1 ml) diluted in phosphate-buffered saline (pH 7·4). A repeat serum sample was obtained from all subjects 2–4 weeks later to assess changes in serum HBV markers due to the skin testing.

Assays of lymphocyte blastogenesis. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque gradient centrifugation. The cells were extensively washed and suspended at a concentration of 1×10^6/ml in culture medium which consisted of RPMI 1640 (MA Bioproducts: Walkersville, Maryland) supplemented with 25 mM HEPES buffer, 10% heat-inactivated fetal calf serum, 4 mM L-glutamine, penicillin and streptomycin (100 μg/ml).

For assays of lymphocyte blastogenesis, 2×10^5 PBMC in 200 μl of medium were cultured in quadruplicate in flat bottomed microtitre wells (Becton Dickinson, Oxnard, California, USA) with or without mitogen for 3 days at 37°C in a humidified atmosphere enriched with 5% CO_2. Final concentrations of mitogens were as follows: 1, 5, 10 and 25 μg/ml of phytohaemagglutinin (PHA)

(Wellcome Research Laboratories, Beckenham, UK); 0·2, 1, 5 and 25 μg/ml of concanavalin A (Con A) (Sigma Chemical Co., St Louis, Missouri, USA); dilutions of 1/500, 1/1,000, 1/10,000 and 1/100,000 of pokeweed mitogen (PWM) (GIBCO, Grand Island, New York, USA); and 0·2, 2 and 4 μg/ml of HBsAg (subtype adw). Four hours before harvest, 1 μCi of ^{3}H-thymidine (New England Nuclear, Boston, Massachusetts) (specific activity 6·7 Ci/mmol) was added to each well. The cells were then harvested onto glass fibre filters for determination of ^{3}H-thymidine incorporation.

The HBsAg used for *in vitro* assays was purified from the plasma of a chronic HBsAg carrier (subtype adw) by two sequential isopycnic ultracentrifugations in caesium chloride, followed by a single rate zonal ultracentrifugation in 10% sucrose and a final rebanding in cesium chloride (Gerin, 1972). Before use, the HBsAg was diluted with and dialysed extensively against culture medium in order to remove caesium chloride. Aggregation of HBsAg particles was avoided by the presence of plasma proteins in the solution. In later studies, PBMC from selected patients were cultured for varying periods of time (3, 5 and 7 days), with higher concentrations of HBsAg (4, 6 and 12 μg/ml) as well as with HBsAg from other sources, including unpurified HBsAg from serum and HBsAg in the HBV vaccine itself.

Statistics. Comparisons between groups were made using the Student's *t*-test for unpaired data.

RESULTS

Skin testing

Delayed hypersensitivity skin test results are summarized in Table 1. All except one subject (a chronic HBsAg carrier) responded to at least one skin test antigen. There were no differences among the groups in percent of subjects responding to any of the skin test antigens or in the average size of the skin test reactions.

Only one subject had a positive delayed skin test reaction to HBsAg. Erythema and induration measured 5 mm at 24 h and 13 mm at 48 h. A biopsy of the skin reaction revealed a dermal mononuclear cell infiltrate and perivenular vasculitis typical of delayed hypersensitivity. Retesting of this individual using different amounts of HBsAg demonstrated a dose–response in the diameter of erythema and induration. At 48 h, induration measured 14 mm with 4 μg, 10 mm with 1 μg, 6 mm with 0·4 μg and < 1 mm with 0·1 μg of HBsAg. This individual had received three injections of HBV vaccine during the preceding year and had high serum titres of anti-HBs (1:3000).

In all the other subjects, including 10 chronic HBsAg carriers, 10 persons who had recovered from type B hepatitis (three within 1–3 months of skin testing) and four vaccine recipients (who had serum titres of anti-HBs ranging from 1:20 to 1:1,000), HBsAg skin testing was negative. In two subjects (one vaccine recipient and one patient recently recovered from type B hepatitis) skin testing with HBsAg caused 2–3 mm of induration at 48 h.

Two subjects with high titres of anti-HBs (one vaccine recipient and one naturally immune

Table 1. Delayed hypersensitivity skin test results

Group	Number tested	Percentage positive With:				
		HBsAg	Mumps	PPD	Candida	Trichophyton
Susceptibles	10	0	100	10	70	0
HBsAg carriers	10	0	90	10	70	10
Vaccinees	5	20	100	40	80	0
Immune individuals	10	0	80	0	60	20
Totals	35	3	91	11*	69	9

*All except one responder to PPD had received BCG in the past.

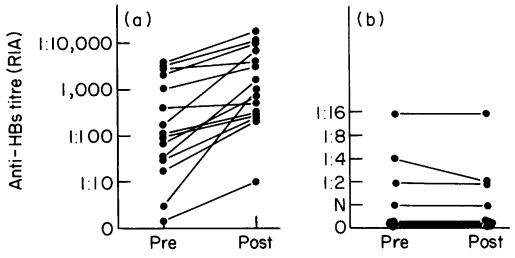

Fig. 1. Titres of antibody to hepatitis B surface antigen (anti-HBs) as detected by radioimmunoassay from (a) 15 immune individuals and (b) 10 chronic HBsAg carriers before (pre) and 2–4 weeks after (post) intradermal skin testing with 4 μg of HBsAg. Titres of anti-HBs increased at least two-fold in all except two of the immune individuals but in none of the chronic HBsAg carriers.

individual) were retested using a five-fold higher concentration of HBsAg. Even with a 20 μg skin test dose, no detectable erythema or induration were present at either 24 or 48 h.

Thirteen of the 15 immune and vaccinated subjects with detectable anti-HBs before skin testing, exhibited a two-fold or greater increase in anti-HBs titres within 2–4 weeks of skin testing (Fig. 1). Four of the ten chronic HBsAg carriers possessed detectable anti-HBs in serum before intradermal skin testing. In each case, the subtype of HBsAg was adw and the subtype specificity of anti-HBs was anti-y. Titres of this monospecific anti-HBs did not increase following skin testing with the HBsAg/adw. The 10 susceptible individuals did not possess anti-HBs before skin testing and remained negative on blood testing 2–4 weeks later (data not shown).

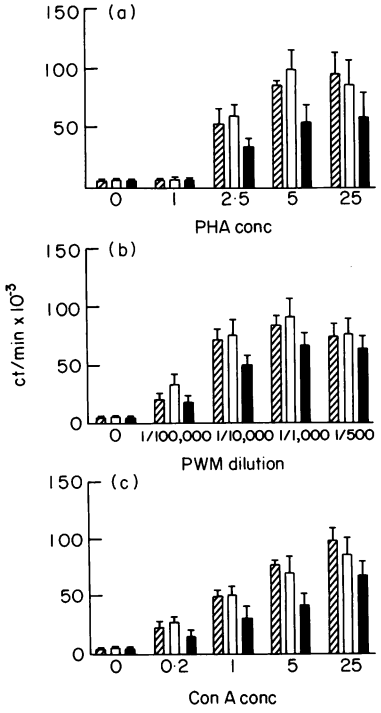

Fig. 2. Results of assays of lymphocyte blastogenesis in response to (a) PHA, (b) Con A and (c) PWM. The ordinate represents the ct/min 3 of H-thymidine uptake by the cultured lymphocytes. The abscissa shows the final concentration of mitogen in the assay. The mean and standard error of the mean are shown for each group: ▨ = susceptible individuals, □ = immune individuals and ■ = chronic HBsAg carriers.

Blastogenesis assays

A summary of results of the lymphocyte blastogenesis assays using non-specific mitogens is shown in Fig. 2. Results from vaccinated and immune individuals (anti-HBs positive) were similar and have been grouped together. There were no appreciable differences between susceptible control individuals and immune persons in the response to any of the mitogens studied. However, the blastogenic responses to each of the three non-specific mitogens was less on average among the chronic HBsAg carriers than among either immune or susceptible individuals. These differences were most marked at intermediate concentrations of mitogens. The largest differences between chronic carriers and control individuals in blastogenic responses were seen using Con A at concentrations of 1 and 5 μg/ml ($P < 0{\cdot}05$).

While the average responses among chronic HBsAg carriers were lower than those among control persons, the individual responses were usually within the range of the normal individuals. Only one of the 10 HBsAg carriers had definitely abnormal blastogenic responses to all mitogens. The person has had negative skin test reactions to all five skin test antigens as well. He was a male homosexual who had had chronic type B hepatitis for 3 years and whose serum was strongly reactive for HBsAg and HBeAg, but whose serum aminotransferase levels were normal (ALT 25 u/l, AST 28 u/l). This patient has developed generalized lymphadenopathy.

Table 2. Lymphocyte blastogenic responses to HBsAg in three groups of patients

Group	Number tested	Mean change in ^{3}H-thymidine incorporation (ct/min) in response to HBsAg concentration (μg)				
		0·2	1·0	4·0	6·0	12·0
Susceptible	10	−400	−300	−900	−800	−1,200
HBsAg carriers	10	−200	−400	−500	Not done	
Immune individuals†	15	−900	+300	+600	−700	−2,800

*Results represent the mean of four wells for each patient studied.
†Both naturally immune and vaccinated persons.

Blastogenic responses to purified HBsAg are shown in Table 2. None of the subjects demonstrated a proliferative response to HBsAg. Whereas, ^{3}H-thymidine incorporation increased by 50,000–100,000 ct/min when lymphocytes were cultured with the non-specific mitogens PHA, Con A and PWM, there was no increase in ^{3}H-thymidine incorporation when lymphocytes were cultured in the presence of HBsAg at concentrations ranging from 0·2 to 12 μg/ml. Lymphocytes from the subject who demonstrated a positive delayed skin test reaction to HBsAg were assayed for blastogenic responses on multiple occasions, for varying periods of time (3–7 days), and using several preparations of purified HBsAg (including the HBV vaccine itself). Despite having delayed hypersensitivity skin test reactivity to HBsAg and a serum anti-HBs titre (after skin testing) as high as 1:10,000, this subject's lymphocytes consistently failed to demonstrate a proliferative response to HBsAg.

DISCUSSION

Cellular immune responses to viral antigens appear to play a major role in the immunopathogenesis of several viral infections, and delayed hypersensitivity skin testing is the most well established means of demonstrating these responses. The role of cellular immunity in the pathogenesis of type B hepatitis has not been well defined. One reason for this is that the viral antigens needed to test for skin test reactivity must be prepared from serum or liver tissue, both of which are highly infectious.

Studies done in guinea-pigs (Gerety, Hoofnagle & Barker, 1974) and chimpanzees (Ibrahim, Vyas & Prince, 1974) have shown that delayed hypersensitivity skin test reactions to HBsAg can be induced by immunization with purified antigen. However, the positive skin test reactions were obtained by using high concentrations of HBsAg (100 μg) or HBsAg in Freund's complete adjuvant, an emulsant that is a potent stimulus to cellular immune responses. In the present study, we were unable to demonstrate skin test reactivity to HBsAg after natural infection in man. Even in hyperimmunized individuals (recent recipients of the HBV vaccine), positive skin test results were rare. The dosage of HBsAg used (4 μg) was one-fifth of the amount generally used to immunize persons with HBV vaccine (20 μg). This is the usual amount of antigen used for delayed hypersensitivity skin testing, i.e. approximately one-tenth of that used for immunization. In addition, retesting of immune individuals with both lower (0·1–1 μg) and higher (20 μg) concentrations of HBsAg failed to demonstrate positive skin test reactions indicating that the negative responses were not due to the use of antigen concentrations that were either too high or too low. Using greater concentrations of HBsAg was not possible because of limitations in the amount of aqueous vaccine available and because of the difficulty in preparing purified HBsAg in a at concentrations greater than 200–300 μg/ml (20–30 μg per 0·1 ml skin test volume).

Another means of demonstrating delayed hypersensitivity to viral antigens is by *in vitro* assays. On the basis of such assays, Dudley *et al.* (1972a) first proposed the hypothesis that cellular immune responses to HBsAg played an important role in recovery from acute type B hepatitis and in the immunopathogenesis of chronic type B hepatitis. In that and subsequent studies (Dudley *et al.*, 1973b; De Moura *et al.*, 1975; Ibrahim *et al.*, 1975; Aldershvile *et al.*, 1977; Lee *et al.*, 1975) leucocyte migration was found to be decreased in the presence of HBsAg in patients with acute and in some patients with chronic type B hepatitis. Similar results have been reported with assays for macrophage inhibition factor (Irwin *et al.*, 1974; Gerber *et al.*, 1974) and lymphocyte blastogenesis to HBsAg (De Gast *et al.*, 1973; Tong *et al.*, 1975; Tiku *et al.*, 1978; Beutner *et al.*, 1978). However, the occasional positive results with these assays on lymphocytes from patients without any serological evidence of HBV infection (Lee *et al.*, 1977; Gerber *et al.*, 1974) raises the question of whether these *in vitro* tests had problems with non-specificity.

The results of the present study differ markedly from those of previous workers who have evaluated lymphocyte blastogenesis to HBsAg. In this study, there were no proliferative responses to HBsAg by lymphocytes from patients with chronic hepatitis or from patients recently recovered from acute type B hepatitis. Cells cultured in the presence of HBsAg usually incorporated less than and in no case incorporated greater than 20% more ^{3}H-thymidine than lymphocytes cultured without HBsAg.

There are several explanations for the differences in results between the present and previously reported studies. These include the number and type of patients studied, the antigen preparation used and its purity, the techniques of lymphocyte isolation and the conditions of the *in vitro* assays themselves.

In this study, most patients were studied during convalescence rather than during the acute illness. In previous studies, cellular immune responsiveness to HBsAg was found to be short lived during acute type B hepatitis. However, cell-mediated responses to other viral, bacterial and fungal antigens are generally long lived (David & David, 1972), and disappearance of delayed hypersensitivity to HBsAg within a few months of acute infection remains unexplained. In this study, the patients with chronic type B hepatitis were mostly male homosexuals, a group that has recently been shown to have significant acquired abnormalities of the immunological system (Mildvan *et al.*, 1982). However, all except one of the patients in this study had normal or near normal proliferative responses to non-specific mitogens and manifested normal skin test reactions to other viral, bacterial and fungal antigens.

The current study employed a highly purified preparation of HBsAg that had been extensively dialysed against culture medium. Previous reported often used partially purified HBsAg and the contribution of other human serum proteins to the proliferative response among patients with acute hepatitis may have played a role in some positive reactions. The current study also employed 'state-of-the-art' techniques of lymphocyte isolation and culture. Previous reports have employed methods that often yield cell preparations that are contaminated with granulocytes.

Finally, and perhaps most importantly, the conditions of the blastogenic assays in this study differed in several ways from previous studies. Yet, despite several manipulations of antigen preparations and concentrations, culture media and duration of cultures, no specific blastogenesis to HBsAg was found. In other studies, not shown here, HBsAg was found not to interfere with proliferation, in that lymphocyte cultures stimulated with PWM in the presence of HBsAg synthesized normal amounts of immunoglobulin (Hanson, Dusheiko & Hoofnagle, 1984).

The lack of lymphocyte proliferation in response to HBsAg in chronic type B hepatitis is in keeping with the characteristic absence of a humoral immune response to HBsAg in this condition (Hoofnagle, 1980; Dusheiko *et al.*, 1983). Indeed, such lymphocyte proliferation in response to HBsAg in patients with this antigen in serum would not be expected. These patients' lymphocytes are constantly exposed to high plasma concentrations of HBsAg (ranging from 0·1 to 500 μg/ml). In performing these assays, the lymphocytes are separated from the HBsAg containing plasma, washed extensively and then placed back into an HBsAg containing medium. A proliferative response in this situation would be more likely due to a contaminant in the HBsAg containing medium or to alterations in the lymphocyte caused by the isolation procedures rather than a specific response to HBsAg.

The results of this study provide no evidence that cell-mediated responses to HBsAg play a major role in the pathogenesis of either acute or chronic type B hepatitis. However, the absence of appreciable cellular immune responses to HBsAg does not mean that cell-mediated immunity plays no role in this disease. It is possible that other HBV antigens such as hepatitis B core antigen or HBeAg may be responsible for the immunological phenomena that accompany type B hepatitis (Gerety *et al.*, 1974; Mondelli *et al.*, 1982). Alternatively, cellular immunity to altered hepatocyte plasma membrane antigens may be important (Meyer zum Büschenfelde *et al.*, 1979). Assessment of delayed hypersensitivity responses to other HBV antigens and to altered hepatocyte antigens is difficult, but deserves further evaluation.

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