Immunology 1987 **62** 593–598

Cellular immunity to nucleocapsid and pre-S determinants in asymptomatic carriers of hepatitis B virus

S. VENTO,* S. -H. CHEN,† G. GIULIANI-PICCARI,‡ C. J. O'BRIEN,* P. R. DAL MONTE,‡ A. L. W. F. EDDLESTON,* C. R. HOWARD† & R. WILLIAMS*
**Liver Unit, King's College School of Medicine and Dentistry, Denmark Hill, †Department of Medical Microbiology, London School of Hygiene and Tropical Medicine, London, and ‡Gastroenterology Unit, Bellaria Hospital, Bologna, Italy*

Accepted for publication 14 August 1987

SUMMARY

Previous studies of cellular immunity in asymptomatic HBV carriers have been limited to evaluation of responses to plasma-derived HBsAg preparations. We have explored the specificity of cellular immune responses to HBV antigens in these subjects using an indirect T-lymphocyte migration inhibitory factor assay and three antigen preparations (recombinant nucleocapsid antigen (HBcAg), plasma-derived HBsAg with or without pre-S2, and *Saccharomyces cerevisiae*-synthesized HBsAg without pre-S2 region). T cells from 10 asymptomatic chronic HBV carriers with normal liver function tests were responsive to nucleocapside determinants (mean migration index = 0·55 ± SD 0·07) and to pre-S2-positive plasma-derived HBsAg (MI = 0·62 ± 0·05). However, none responded to HBsAg devoid of pre-S2 sequences (MI = 0·98 ± 0·04). In further experiments, T cells from three HBV carriers, cultured with six different HBsAg preparations, exhibited responsiveness only to those preparations containing significant pre-S2 activities. Our results show that T-cell immunity to nucleocapsid determinants of the virus and HBsAg in present in asymptomatic HBV carriers; the latter is restricted to antigenic preparations containing significant pre-S2 activities. Hence, T-cell immunity to pre-S determinants may not always be associated with HBV clearance.

INTRODUCTION

The development of a chronic carrier state following infection with hepatitis B virus (HBV) is a problem of worldwide importance (Blumberg, Sutnick & London, 1971) because of the associated increased risk of cirrhosis and hepatocellular carcinoma. However, the reasons why the virus fails to be eliminated in these individuals remain unclear. It has long been suspected that HBsAg carriers, particularly if asymptomatic, are unable to mount an immune response to HBsAg (Dudley, Fox & Sherlock, 1972). Most *in vitro* studies of cellular reactivity to HBsAg support this view, although recently an *in vitro* cellular immune response has been demonstrated using very low concentrations of HBsAg and purified T cells from asymptomatic HBsAg carriers (Sylvan, Hellström & Lundbergh, 1985). The antigenic determinants involved in this response were not identified and it is unclear whether they were part of HBsAg itself, or an associated component. For example, a newly recognized component of the viral envelope coded for by the pre-S region of HBV DNA (Machida *et al.*, 1983) and carrying receptors for polymerized human serum albumin (pHSA) (Alberti *et al.*, 1984) is now known to be present in some preparations of HBsAg obtained from serum. Immune responses to this receptor could contribute to neutralization of hepatitis B virions since it has been suggested that pHSA bound to the receptor may facilitate the entry of virus into liver cells (Thung & Gerber, 1983).

In the present study, we have explored the specificity of cellular immune responses to HBV antigens in asymptomatic carriers using an indirect T-lymphocyte migration inhibitory factor assay (Vento *et al.*, 1984, 1985) with four different purified antigen preparations. Hepatitis B nucleocapsid antigen was prepared from *E. coli* carrying a recombinant plasmid containing the HBcAg-specific gene (Pasek *et al.*, 1979). Three sources of HBsAg particles were used. The first was antigen purified from the plasma of e antigen-positive carriers of HBV containing S envelope determinants, together with variable amounts of pre-S2. The second was purified from culture supernatants of PLC/PRF/5 hepatoma cells and contained trace amounts of pre-S2 sequences. The third was expressed in *Saccharomyces cerevisiae* and contained S determinants only.

Correspondence: Professor A. L. W. F. Eddleston, The Liver Unit, King's College Hospital, Denmark Hill, London SE5 9RS U.K.

MATERIALS AND METHODS

Subjects

Ten asymptomatic HBsAg carriers (seven females; mean age 34 years, range 12–56 years) were studied. They had no detectable

anti-HBs in serum and possessed titres of HBsAg in the range 1:3200 to 1:12,800, as determined by haemagglutination inhibition. Antibodies to hepatitis B core antigen (anti-HBc) were present, in titres from 1:1000 to 1:10,000 (CORAB, Abbott Laboratories, North Chicago, IL). Antibodies to hepatitis B e antigen (anti-HBe) were present in seven subjects, and the remaining three were HBeAg +ve. Percutaneous liver biopsies were available in five carriers and showed normal histology. All ten patients had been closely followed over a 2–15-year period, and their clinical status and liver function tests (AST, ALT, alkaline phosphatase and γ-GT), serum proteins and immunoglobulins were consistently found to be normal before inclusion in the study. Ten volunteers seronegative for HBV with normal liver function tests, ESR, blood picture and urinanalysis (five females; mean age 31 years, range 19–47 years) were tested as controls.

Serological tests and antigens

Hepatitis B serology was analysed by commercial kits (AUSRIA II, AUSAB, CORAB, HBeAg and anti-HBe Diagnostics, Abbott Laboratories).

HBsAg particles were purified from plasma by centrifugation and gel chromatography, essentially as described by Skelly, Howard & Zuckerman (1978). Six separate lots of plasma-derived antigen were used, each purified from different hepatitis B carriers. The final concentration of HBsAg in each preparation was determined by assuming an 0·1 extinction coefficient at 280 nm of 37. HBsAg particles from PLC/PRF/5 cells were purified from 10-day-old cultures by differential and equilibrium centrifugation.

The HBsAg particles containing S envelope determinants alone were produced in fermentation cultures of *Saccharomyces cerevisiae* carrying an expression vector using yeast alcohol dehydrogenase I as a promoter (Murray *et al.*, 1984). Cells were collected by centrifugation and broken down by homogenization with glass beads. HBsAg particles were purified from the clarified extract by affinity chromatography using goat antibodies to human HBsAg. Electron microscopy demonstrated an homogeneous array of HBsAg particles free of extraneous morphological entities. SDS–polyacrylamide gel electrophoresis revealed a major band of molecular weight 25,000 corresponding to the non-glycosylated major polypeptide of the viral envelope. The final HBsAg preparation was adjusted to a concentration of 0·25 mg/ml in 0·02 M Tris-HCl, pH 7·8, containing 5% sucrose.

Bacterial extracts of *E. coli* K 12 strain HB 101 containing the recombinant plasmid pHBV-R1-II carrying the HBcAg-specific gene were prepared as previously described (Pasek *et al.*, 1979). Both HBcAg and HBeAg determinants are present on this preparation of recombinant nucleocapsid antigen (Mackay, Lees & Murray, 1981). The protein concentration was adjusted by spectrophotometry to 2·5 mg/ml, which corresponded to an estimated HBcAg concentration of 1·25 mg/ml as assessed by radioimmunoassay.

Polymerized human serum albumin (pHSA) binding activity of HBsAg

The PHSA binding activity of HBsAg was measured by direct ELISA assay, as described by S. H. Chen and C. R. Howard (submitted for publication). Briefly, human serum albumin (HSA, Calbiochem-Behring Corp, San Diego, CA) was dissolved in 0·16 M phosphate buffer (pH 8·6) and polymerized with 0·25% glutaraldehyde at room temperature for at least 2 hr with occasional mixing until the solution became opalescent. The solution (pHSA) was dialysed against PBS and adjusted to an albumin concentration of 500 μg/ml with PBS. Fifty microlitres of freshly diluted pHSA (50 μg/ml in 0·1 M carbonate buffer) were coated onto wells of micro-ELISA plates (Dynatech, Billinghurst, Sussex). These were designated as pHSA wells. The same concentration of unpolymerized albumin (HSA) was also coated onto the surface of control wells of each plate. Fifty microlitres of purified HBsAg, diluted with PBS, were added to the PHSA/HSA wells. After 2 hr of incubation at room temperature, the wells were washed five times with PBS/Tween. One hundred microlitres of mouse ascitic monoclonal antibody IgG against HBsAg 'a' determinant conjugated with horseradish peroxidase were added. The plate was incubated at room temperature for another 2 hr, and then washed five times with PBS/Tween. One hundred microlitres of the substrate solution (0·04% *o*-phenylenediamine and 0·024% w/v hydrogen peroxide in 0·05 M citrate buffer containing 0·1 M disodium phosphate, pH 5·0) were added. The reaction was stopped with 50 μl of 4 N sulphuric acid and the optical density was then measured at 490 nm. The specific value of PHSA-binding activity of HBsAg was obtained by subtracting the reading of a HSA-coated well from that of an equivalent PHSA-coated well.

Polypeptide composition analysis of HBsAg

Polypeptide composition analysis of spherical HBsAg particles was done by discontinuous SDS–PAGE technique, mainly according to Young *et al.* (1982). Briefly, HBsAg particles were disrupted at 100° for 3 min in the disruption buffer (0·04 M Tris–0·022 M phosphate buffer, pH 6·6, containing 2% SDS, 2% 2-mercaptoethanol, 10% glycerol and 0·002% bromophenol blue), and then applied to a 7·5–15% linear acrylamide gradient gel slab (1·5 mm thick, 14 cm wide and 12 cm high). The linear gradient resolving gel was prepared from a 28·6% (w/v) acrylamide stock solution containing 1·4% (w/v) *NN'*-methylene bis-acrylamide and buffered with 0·3 M Tris–HCl, pH 8·8, and 10% glycerol. The stacking gel consisted of 3·3% acrylamide and 0·16% bis-acrylamide buffered with 0·06 M Tris–0·03 M phosphate buffer, pH 6·6. The gel was electrophoresed overnight at 50 V in 0·07 M Tris buffer, pH 8·4, containing 0·38 M glycine and 0·1% SDS. The BioRad electrophoresis chamber (Model PROTEAN 16 cm) and LKB gradient former were used. After the run, the slab gel was stained with 0·24 (w/v) Coomassie brilliant blue R (dissolved in fixing solution, methanol:glacial acetic acid:H_2O = 64:23:64 by volume) and subsequently superstained with silver stain according to the instructions of the BioRad silver stain kit. The low molecular weight calibration kit (Pharmacia Fine Chemicals, Uppsala, Sweden) was used as marker proteins. For immunoblotting analysis, the unstained gel was transferred to the BioRad Trans-Blot nitrocellulose paper by electrophoresis in BioRad Trans-Blot cell at 0·3 A for 4 hr. Tris buffer (0·025 M) containing 0·192 M glycine and 15% (v/v) methanol, pH 8·3 was used as electrophoresis buffer (Heerman *et al.*, 1984). The paper was washed twice with PBS/Tween and incubated with 0·5% gelatin/PBS at room temperature with constant shaking for at least 1 hr. The paper was washed five times and then blotted with pHSA (140 μg/ml in PBS, prepared as above) or rabbit anti-HBsAg antiserum (Behring Institute, 1:400 in PBS) to show either pHSA-binding

proteins or all of the HBsAg-specific polypeptide bands, respectively. After 2 hr of incubation at room temperature, the blot was washed five times with PBS/Tween. The bound anti-HBsAg antibodies were detected by 2 hr further of incubation with goat anti-rabbit IgG–peroxidase conjugate (Miles Scientific, 1:5000 in PBS/Tween) at room temperature. The blot was then washed again similarly and incubated with the substrate solution (0·05 M Tris–HCl buffer, pH 7·5, containing 0·01% w/v diaminobenzidine and 0·06% w/v hydrogen peroxide). The reaction was stopped by washing with PBS/Tween. The bound PHSA was detected by incubation with rabbit anti-human serum albumin antisera (Miles Scientific, Slough, Berks, 1:500 in PBS) at room temperature for 2 hr and subsequently with goat anti-rabbit IgG–peroxidase conjugate.

Preparation of T lymphocytes

An enriched T-cell population was prepared as previously described (Vento *et al.*, 1984, 1985). Briefly, mononuclear cells were obtained from peripheral blood by the fractionation of leucocyte-enriched plasma over a Ficoll–Triosil gradient (Pharmacia Fine Chemicals) followed by depletion of plastic-adherent cells. The recovered population was washed and resuspended at 4×10^6 cells/ml in RPMI and 20% fetal calf serum. Four-millilitre volumes were incubated with equal volumes of 1% sheep red blood cells for 2 hr at 4°, and the pellet, obtained by centrifugation through a Ficoll–Triosil gradient, was treated with 0·83% ammonium chloride to lyse the sheep red blood cells. This pelleted population contained 92–96% E-rosette forming cells, 1–1·5% monocytes and 2·5–7% B and null cells. T-lymphocyte enriched populations were incubated for 48 hr with and without HBcAg (7 μg/ml) or HBsAg (10 μg/ml) at a concentration of 3×10^6 cells/ml in RPMI supplemented with 1% fetal calf serum. Cell-free supernatants were harvested by centrifugation at 900 ***g*** for 10 min and stored at −20° until assayed.

Indirect T-lymphocyte migration inhibitory factor test

This assay, in which T-cell immunity to an antigen is detected by the release of factor(s) (T-LIF) inhibiting the migration of normal T lymphocytes out of agarose microdroplets, has been described previously (Vento *et al.*, 1984, 1985). T cells prepared from a normal, unresponsive individual were suspended in 0·2% agarose and RPMI containing 10% fetal calf serum at a concentration 2×10^8 cells/ml and dispensed in 2-μl drops into the wells of a flat-bottomed microtitre test plate. One hundred-microlitre aliquots of the supernatants obtained from T-cell cultures were added to triplicate wells and the mean area of migration measured by planimetry after 20 hr of incubation at 37°. A migration index was then calculated as the ratio of cell migration in the presence of supernatants obtained from cultures of T cells with and without antigen. A migration index of less than 0·80 was taken, as previously (Vento *et al.*, 1984, 1985), to indicate the presence of significant T-LIF activity and thus T-cell immunity to the antigen used in the assay.

RESULTS

The HBsAg preparations were screened initially for pHSA-binding activity as a measure of the presence of pre-S2 determinants. PHSA binding was clearly detected in preparations 1, 2, 4 and D3, all derived from the plasma of hepatitis B carriers. Lot 3, together with HBsAg from the hepatoma cell line, contained trace amounts of pre-S2 determinants, and lot 6 and the yeast-derived antigen were negative (Table 1 and Fig. 1).

More HBsAg preparations from carriers of different eAg/eAb status have been analysed and the existence of high PHSA-binding activity as well as pre-S2 content appears closely correlated with the eAg positivity (S. H. Chen and C. R. Howard, submitted for publication). Moreover, the original carrier's plasma of lot 3 was found to have high titres of antibodies against both pre-S1 and pre-S2 antigens (Mackay *et al.*, 1981). This may explain why lot 3 HBsAg contains far fewer pre-S2 proteins (designated as ± in Table 1).

T lymphocytes from all the 10 asymptomatic HBsAg carriers included in this study generated T-LIF activity when cultured with nucleocapsid antigen (MI = 0·55 ± SD 0·07) and serum-derived HBsAg, D3 (MI = 0·62 ± 0·05). However, none responded to HBsAg devoid of pre-S sequences (MI = 0·98 ± 0·04) (Fig. 2). T-LIF activity was not generated by the T cells from any of the 10 normal subjects seronegative for all markers of HBV in the presence of either serum-derived HBsAg, yeast-derived HBsAg or nucleocapsid antigen (MI = 0·95 ± 0·06; 0·94 ± 0·04, and 0·94 ± 0·07, respectively) (Fig. 2).

In further experiments, the T lymphocytes from three of the asymptomatic HBsAg carriers generated T-LIF activity in the presence of antigen from D3, nucleocapsid antigen and three additional preparations of serum-derived HBsAg (lots 1, 2 and 4), while the four remaining preparations of HBsAg did not stimulate any T-LIF release (lots 3 and 6 from plasma, lot 5 from hepatoma cells, and yeast-derived HBsAg) (Fig. 3).

DISCUSSION

The present results demonstrate that asymptomatic HBsAg carriers have circulating T lymphocytes reactive to nucleocapsid determinants of HBV. Our results also strongly suggest the presence of T-cell populations responsive to some, but not all, envelope antigens present on the surface of HBsAg particles.

While cellular immunity to nucleocapsid determinants has not previously been assessed in asymptomatic carriers, a large number of studies has been reported using HBsAg as antigen in various *in vitro* systems (Laiwah, 1971; Tong *et al.*, 1975; Ibrahim, Vyas & Perkins, 1975; Tiku *et al.*, 1978; Hanson *et al.*, 1984; Sylvan *et al.*, 1985). In all but one (Sylvan *et al.*, 1985), no significant response was detected. However, the cell populations used were almost always markedly heterogeneous, not only containing uncontrolled proportions of monocytes and B and T lymphocytes (Laiwah, 1971; Tong *et al.*, 1975; Hanson *et al.*, 1984) but, in some studies, polymorphs (Ibrahim *et al.*, 1975) and even erythrocytes (Tiku *et al.*, 1978). This may well have affected the results, and it is of interest that when purified T lymphocytes, the main effector cells of cellular immunity, have been used in both this and a previous study of asymptomatic HBsAg carriers (Sylvan *et al.*, 1985), the presence of cell-mediated immunity to HBsAg has been demonstrated. However, our findings also show that T-cell immunity to HBsAg is restricted to antigenic determinants exclusively present on preparations of HBsAg containing a high concentration of pre-S sequences, and it may have been differences in the HBsAg preparations used in the previous studies that determined the findings.

Table 1. Characterization of HBsAg preparations used in this study

HBsAg	eAg status of carriers: eAg	eAg status of carriers: eAb	pHSA binding activity*	Presence of pre-S(2)-containing polypeptide detected by:† Silver staining	Immunoblotting with: pHSA	Immunoblotting with: Anti-HBsAg
Sera:						
Lot 1	+	−	3	+	+	+
Lot 2	+	−	3	+	+	+
Lot 3	−	+	2	−	±	±
Lot 4	+	−	4	+	+	+
Lot 6	−	−	1	−	−	−
D3	+	−	3	+	+	+
Yeast			0	−	−	−
PLC/PRF/5:						
Lot 5			0–1	−	−	±

± = occasionally weakly positive.

*10 μg/ml of HBsAg were tested in pHSA/HSA-coated wells as shown in the Materials and Methods; smaller numbers represent lower activity.

† Analysed by SDS–PAGE followed by silver staining or immunoblotting.

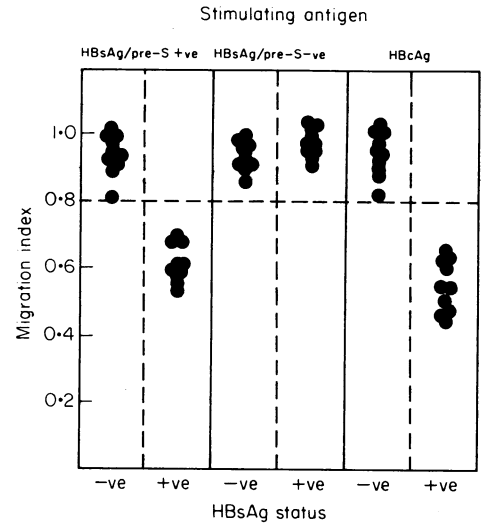

Figure 2. Migration indices representing T-LIF release from T lymphocytes. Different preparations of T cells were purified from 10 asymptomatic HBV carriers or 10 HBV-seronegative normal controls, and activated with serum-derived HBsAg lot D3 (pre-S2-positive), yeast-expressed HBsAg (pre-S2-negative), or recombinant nucleocapsid antigen (HBcAg). Migration indices below 0·8 are considered positive.

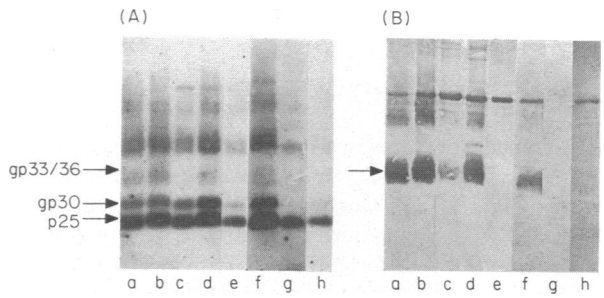

Figure 1. Polypeptide composition analysis of HBsAg. HBsAg preparations were disrupted by SDS and 2-mercaptoethanol and applied to the 7·5–15% acrylamide linear gradient slab gel. The electrophoresed gel was then transferred to nitrocellulose paper and either immunoblotted with rabbit anti-HBsAg antiserum (A) or pHSA (B). The gp33/gp36 band represents the pre-S2-containing polypeptides. Lanes a, b, c, d, e and f represent serum-derived HBsAg lots 1, 2, 3, 4, 6 and D3, respectively. Lanes g and h represent yeast-expressed and PLC/PRF/5-derived HBsAg lot 5, respectively.

The S gene of HBV codes for the major 226 amino-acid long envelope polypeptide of molecular weight 25,000, the predominant component of the yeast-derived HBsAg preparation used in this study. Transcription of this gene can be initiated from at least one, possibly two, initiation codons upstream from the S gene in the so-called pre-S region. This generates additional amino-terminal peptides termed pre-S2 and pre-S1, respectively, according to length. These may be particularly prominent in HBsAg preparations from carriers supporting high levels of virus replication (S. H. Chen and C. R. Howard, submitted for publication; Stibbe & Gerlich, 1983). Both pre-S1 and pre-S2

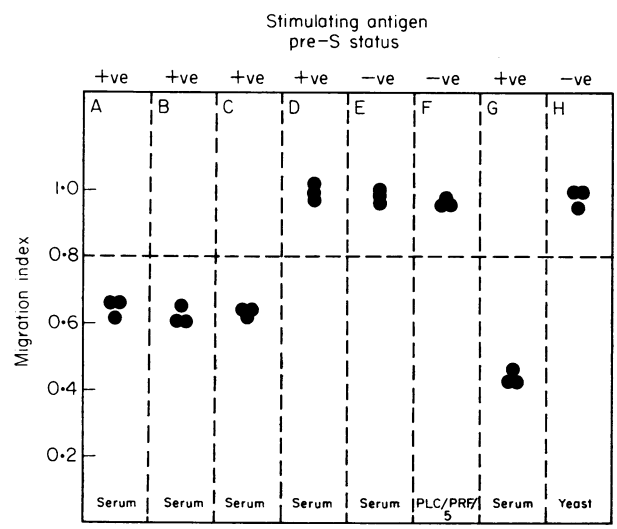

Figure 3. Migration indices representing T-LIF release from T lymphocytes prepared from three of the asymptomatic HBV carriers and activated with serum-derived HBsAg lots 1, 2, 4 and D3 (pre-S2-positive, lanes A, B, C and G, respectively), lot 3 (weakly pre-S2-positive, lane D), lot 6, PLC/PRF/5-derived HBsAg lot 5, and yeast-expressed HBsAg (pre-S2-negative, lanes E, F and H, respectively). Migration indices below 0·8 are considered positive.

sequences are believed to represent functionally important gene products and may be important for mediating the attachment of HBV to hepatocytes (Machida *et al.*, 1983; Neurath *et al.*, 1985, 1986).

Whether the observations in this study are relevant to the defects in the clearance of HBV responsible for the carrier state is not yet clear. Since cellular unresponsiveness to S-encoded HBsAg is common to carriers with (Vento *et al.*, 1985) and without liver damage, it is tempting to speculate that this could be one of the causes of viral persistence. On the other hand, recent attempts to explain the process of viral elimination have focused on the importance of a cytolytic T-cell response to nucleocapsid determinants of HBV, serologically defined as HBcAg (Mondelli *et al.*, 1982; Naumov *et al.*, 1984) or HBeAg (Pignatelli *et al.*, 1987), on the surface of infected hepatocytes, and have suggested that the elimination of released virions is more likely to be due to an antibody response to pre-S2 and pre-S1 determinants (Mondelli & Eddleston, 1984), rather than to HBsAg, since anti-HBs is detected so late in the recovery phase of an uncomplicated acute hepatitis B. In contrast, antibodies to pre-S region-encoded components of the envelope of hepatitis B virions are well established at clinical presentation (Alberti *et al.*, 1978, 1984). The present finding of T-cell immunity to pre-S determinants in some carriers is apparently at variance with this concept that an immune response to the pre-S region is important in viral clearance. However, in a non-lytic infection, clearance of virus-infected cells is as important as virus neutralization in eradicating infection, and in the present study only delayed-type hypersensitivity T-cell immune responses have been evaluated, which may not be accompanied by cytolytic T-lymphocyte activation. The absence of liver damage in asymptomatic carriers strongly suggests that there is a significant defect in T-cell cytolytic mechanisms in these patients. As yet, the nature of this defect has not been determined, but these results emphasize that recovery from HBV infection is more complex than simply making an immune response to pre-S determinants, and suggest that it would be unwise to rely entirely on a pre-S vaccine for complete protection against HBV.

ACKNOWLEDGMENT

We are most grateful to Professor K. Murray, University of Edinburgh, for the generous donation of the HBsAg particles containing S envelope determinants.

REFERENCES

ALBERTI A., DIANA S., SCULLARD G.H., EDDLESTON A.L.W.F. & WILLIAMS R. (1978) Detection of a new antibody system reacting with Dane particles in hepatitis B virus infection. *Br. Med. J.* **2,** 1056.

ALBERTI A., PONTISSO P., SCHIAVON E. & REALDI G. (1984) An antibody which precipitates Dane particles in acute hepatitis B: relation to receptor sites which bind polymerised human serum albumin on virus particles. *Hepatology*, **4,** 220.

BLUMBERG B.S., SUTNICK A.I. & LONDON W.T. (1971) Australia antigen and hepatitis: a comprehensive review. *CRC Crit. Rev., Clin. Lab. Sci.* **2,** 473.

DUDLEY F.J., FOX R.A. & SHERLOCK S. (1972) Cellular immunity and hepatitis-associated Australia antigen liver disease. *Lancet*, **i,** 723.

HANSON R.G., HOOFNAGLE J.H., MINUK G.Y., PURCEL R.H. & GERIN J.L. (1984) Cell-mediated immunity to hepatitis B surface antigen in man. *Clin. exp. Immunol.* **57,** 257.

HEERMAN K.H., GOLDMANN U., SCHWARTZ W., SEYFFARTH T., BAUMGARTEN H. & GERLICH W.H. (1984) Large surface proteins of hepatitis B virus containing the pre-S sequence. *J. Virol.* **52,** 396.

IBRAHIM A.B., VYAS G.N. & PERKINS H.A. (1975) Immune response to hepatitis B surface antigen. *Infect. Immun.* **11,** 137.

LAIWAH A.A.C.Y. (1971) Lymphocyte transformation by Australian antigen. *Lancet*, **ii,** 470.

MACHIDA A., KISHIMOTO S., OHNUMA H., MIYAMATO H., BABA K., ODA K., NAKAMURA T., MIYAKAWA Y. & MAYUMI M. (1983) A hepatitis B surface antigen polypeptide (P31) with the receptor for polymerised human as well as chimpanzee albumins. *Gastroenterology*, **85,** 268.

MACKAY P., LEES J., MURRAY K. (1981) The conversion of hepatitis B core antigen synthesized in *E. coli* into e antigen. *J. med. Virol.* **8,** 237.

MONDELLI M. & EDDLESTON A.L.W.F. (1984) Mechanisms of liver cell injury in acute and chronic hepatitis B. *Semin. Liver Dis.* **4,** 47.

MONDELLI M., MIELI-VERGANI G., ALBERTI A., VERGANI D., PORTMANN B., EDDLESTON A.L.W.F. & WILLIAMS R. (1982) Specificity of T-lymphocyte cytotoxicity to autologous hepatocytes in chronic hepatitis B virus infection: evidence that T-cells are directed against HBV core antigen expressed on hepatocytes. *J. Immunol.* **129,** 2773.

MURRAY K., BRUCE S.A., HINNON A., WINGFIELD P., VAN ERD P.M., DE REUS A. & SCHELLEKENS H. (1984) Hepatitis B virus antigen made in microbial cells immunised against viral infection. *EMBO J.* **3,** 645.

NAUMOV N.V., MONDELLI M., ALEXANDER G.J.M., TEDDER R.S., EDDLESTON A.L.W.F. & WILLIAMS R. (1984) Relationship between expression of HBV antigens in isolated hepatocytes and autologous lymphocyte cyotoxicity in patients with chronic HBV infection. *Hepatology*, **4,** 63.

NEURATH A.R., KENT S.B.H., STRICK N. & PARKER K. (1986) Identification and chemical synthesis of a host cell receptor binding site on hepatitis B virus. *Cell*, **46,** 429

NEURATH A.R., KENT S.B.H., STRICK N., TAYLOR P. & STEVENS C.E. (1985) Hepatitis B virus contains pre-S gene-encoded domains. *Nature (Lond.)*, **315,** 154.

PASEK M., GOTO T., GILBERT W., ZINK B., SCHALLER H., MACKAY P., LEADBETTER G. & MURRAY K. (1979) Hepatitis B virus genes and their expression in *E. coli. Nature (Lond.)*, **282,** 575.

PIGNATELLI M., WATERS J., LEVER A., IWARSON S., GERETY R. & THOMAS H.C. (1987) Cytotoxic T-cell responses to the nucleocapsid proteins of HBV in chronic hepatitis. Evidence that antibody modulation may cause protracted infection. *J. Hepatol.* **4,** 15.

SKELLY J., HOWARD C.R. & ZUCKERMAN A.J. (1978) The labelling of galactose residues in hepatitis B surface antigen glycoprotein. *J. gen. Virol.* **41,** 447.

STIBBE W.R. & GERLICH W. (1983) Structural relationships between minor and major proteins of hepatitis B surface antigen. *J. Virol.* **46,** 626.

SYLVAN S.P.E., HELLSTRÖM U.B. & LUNDBERGH P.R. (1985) Detection of cellular and humoral immunity to hepatitis B surface antigen (HBsAg) in asymptomatic HBsAg carriers. *Clin. exp. Immunol.* **62,** 288.

THUNG S.N. & GERBER M.A. (1983) Hepatitis B virus and polyalbumin binding sites. *Gastroenterology*, **85,** 466.

TIKU M.L., BEUTNER K.R., TIKU K. & OGRA P.L. (1978) Cell-mediated immune response to liver tissue antigen and hepatitis B surface antigen after infection with hepatitis B virus in humans. *J. infect. Dis.* **138,** 587.

TONG M.J., WALLACE A.M., PETERS R.L. & REYNOLDS T.B. (1975) Lymphocyte stimulation in hepatitis B infection. *N. Engl. J. Med.* **293,** 318.

VENTO S., HEGARTY J.E., ALBERTI A., O'BRIEN C.J., ALEXANDER G.J.M., EDDLESTON A.L.W.F. & WILLIAMS R. (1985) T-lymphocyte sensitisation to hepatitis B core Ag and T-cell-mediated unresponsive-

ness to hepatitis HBsAg in hepatitis B virus-related chronic liver disease. *Hepatology*, **5**, 192.

VENTO S., HEGARTY J.E., BOTTAZZO G.F., MACCHIA E., WILLIAMS R. & EDDLESTON A.L.W.F. (1984) Antigen-specific suppressor cell function in autoimmune chronic active hepatitis. *Lancet*, **i**, 1200.

YOUNG P.R., VAUDIN M., DIXON J. & ZUCKERMAN J.A. (1982) Preparation of hepatitis B polypeptide micelles from human carrier plasma. *J. virol. Meth.* **4**, 177.