Virus-neutralizing Antibodies to Hepatitis B Virus: The Nature of an Immunogenic Epitope on the S Gene Peptide

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SUMMARY

Using a murine monoclonal antibody (RF-HBs-1) which has been shown to be capable of neutralizing both ad and ay subtypes of hepatitis B virus (HBV), we have devised a competitive inhibition assay to measure the presence of virus-neutralizing antibodies in the sera of patients who have recovered from acute type B hepatitis. The majority of patients have this antibody in their serum. We also show that this antibody inhibits the binding of polymerized human serum albumin (pHSA) to the pHSA receptor site of the HBV particle, which has been proposed as an important site for the entry of HBV into liver cells. We have demonstrated that the epitope recognized by this antibody is dependent on the linkage of 24000 and 28000 mol. wt. polypeptides via a disulphide bond. This conformational determinant in the coat of the virus which is part of or near to the pHSA binding site is important in evoking a virus-neutralizing response.

INTRODUCTION

Hepatitis B surface antigen (HBsAg) is part of the outer coat of the hepatitis B virus (HBV). This glycoprotein also exists in the form of 22 nm diameter spherical and tubular structures which do not contain viral DNA. Following natural infection there is a humoral immune response to the HBsAg which, in most cases, confers protective immunity. In addition, purified HBsAg particles derived from plasma of persistently infected individuals (Szmuness et al., 1980) or produced in yeast by recombinant DNA technology (Jilg et al., 1984), have been used successfully as a vaccine against this infection. The proteins of the HBsAg have been characterized. HBsAg, when disrupted under reducing conditions, has been shown to consist of two major polypeptides of mol. wt. 20000 to 25000 (P24) and 25000 to 30000 (GP28) and several minor polypeptides of higher molecular weight (Peterson, 1981; Zuckerman & Howard, 1979; Dreesman et al., 1975). These two polypeptides have the same primary amino acid sequence and represent non-glycosylated and glycosylated forms of the basic peptide of HBsAg (Peterson, 1981). Both bear the common 'a' determinant present on all particles and known to induce protective antibody responses in man (Szmuness et al., 1982).

Receptors for polymerized human serum albumin (pHSA) have been identified on HBV by several groups (Imai et al., 1979; Hansson & Purcell, 1979). Similar receptors have been identified on the surface membrane of hepatocytes (Lenkei et al., 1977; Trevisan et al., 1982) and it has been suggested that these receptors could be important in HBV infection by facilitating entry of the virus into the hepatocyte. Antibodies which react with the pHSA receptor may then be important in virus neutralization. Initial studies revealed polyalbumin receptors on HBsAg polypeptides of mol. wt. 22000 and 68000 (Ionesco-Matiu et al., 1980). Another group have subsequently identified polyalbumin receptor activity on the 31000 mol. wt. pre-S/S polypeptide (Machida et al., 1983) which is present in greatest concentration in HBV particles (Stibbe & Gerlich, 1982).
The P24 and GP28 polypeptides when isolated individually were immunogenic in guinea-pigs, but to a lesser degree than the intact particles (Cabral et al., 1978). However, a 49000 mol. wt. polypeptide prepared by treating HBsAg particles with SDS and composed of one P24 and one GP28 polypeptide of HBsAg retained the same antigenicity as the intact particles (Mishuro et al., 1980). Furthermore, the immunogenicity of micelles formed from the polypeptides extracted from 22 nm HBV particles using Triton X-100 was higher than that of intact 22 nm particles in mice (Skelly et al., 1981). These data suggest that the immunogenicity of HBsAg is dependent on the association of the two major polypeptides.

It is now well established that the humoral immune response to the coat proteins of a virus will include a variety of antibody molecules reacting with different epitopes on the surface of the virus particle. Some of these antibodies will be virus-neutralizing and some will not. In order to determine which epitopes on HBV are important in stimulating virus-neutralizing antibody responses, we have produced a series of murine monoclonal antibodies to this protein and identified one in chimpanzees, which is capable of neutralizing both ad and ay subtypes of the virus (Iwarson et al., 1985). In this paper, we show that the antibody evoked by this epitope is found in the majority of patients who have recovered from HBV, that it inhibits HBV particle binding to pHSA and that it binds to a conformational determinant formed by the disulphide bonding of the P24 and GP28 polypeptides.

**METHODS**

**RF-HBs-1 inhibition assay.** The production and characterization of the murine monoclonal antibody to HBsAg (RF-HBs-1) has been described previously (Goodall et al., 1981). The antibody was purified from ascitic fluid using a Protein A-Sepharose 4-B column (Pharmacia).

Purified RF-HBs-1 (10 μg) was radiolabelled with 20 MBq sodium [125I]iodide (Amersham) using iodogen, 10 μg/tube in chloroform (Pierce Chemical, Rockford, Ill., U.S.A.). The reaction was carried out for 15 min at room temperature. The labelled antibody was separated from unbound [125I] using a 1 ml Dowex-1 column equilibrated in phosphate-buffered saline (PBS) pH 7.2 containing 0.1% bovine serum albumin (BSA; Sigma). A competitive inhibition assay was used to detect RF-HBs-1-like antibodies in human serum. HBsAg for use on the solid phase was purified from the serum of chronic carriers of HBV using an affinity column of RF-HBs-2 (Goodall et al., 1981), which binds to an epitope distinct from that recognized by RF-HBs-1 coupled to cyanogen bromide-activated Sepharose 4-B (Pharmacia). The HBsAg was absorbed onto polystyrene beads (Northumbria Biologicals) for use as a solid phase by diluting the antigen, at an optimum concentration, in coating buffer (0.035 M-sodium bicarbonate, 0.015 M-sodium carbonate, 0.02% sodium azide) and mixing with the beads for 1 h at room temperature and overnight at 4 °C. The solid phase was co-incubated with 100 μl of test serum and 100 000 c.p.m. radiolabelled RF-HBs-1 overnight at room temperature. Sera from patients who had recovered from acute type B hepatitis and who were HBs antibody-positive by AUSAB (Abbott Laboratories), and normal human serum negative for HBs antibody by the same assay, were examined in this competitive inhibition assay.

**pHSA binding assay.** Globulin-free human serum albumin (HSA) (Behringwerke, F.R.G.) was polymerized according to the method of Lenkei et al. (1977). Briefly, 30 mg HSA in 0.9 ml 0.1 M-phosphate buffer pH 6.8, was incubated with 0.1 ml 25% glutaraldehyde solution for 2 h at room temperature, and then dialysed for 3 h against several changes of PBS pH 7.2. The sample was run through a G-200 Sephadex column and the leading peak was collected, concentrated and stored at −20 °C.

HBV particle-enriched HBsAg was purified by ultracentrifugation of serum from a chronic HBV carrier who was HBe antigen-positive. Two-hundred ml serum was clarified at 5000 r.p.m. for 30 min and the supernatant was further centrifuged at 37000 r.p.m. for 3 h (Sorvall A841 rotor, Sorval 65 OTD centrifuge). The pellet was resuspended in 2 ml TNEM–BSA buffer (0.05 M-Tris–HCl pH 7.4, 0.16 M-NaCl, 0.1% BSA) and stored at −20 °C. The same preparation of Dane particle-enriched HBV was used for all experiments. The amount of HBsAg was assayed against the British Standard (ref. no. 80/549: The National Biological Standards Board, London) using an assay described previously (Goodall et al., 1981).

Five μg HBsAg was radiolabelled using the iodogen method as used for the monoclonal antibody. The bound iodine was separated from the free by dialysing against several changes of PBS (pH 7.2).

The inhibition of pHSA binding to HBsAg (containing HBV particles) was measured by radioimmunoassay. Microtitre plates (Flow Laboratories) were coated with 50 μl pHSA (50 μg/ml) overnight at room temperature, and blocked with 5% BSA for 3 h at 4 °C. Twenty-five μl of a known concentration of purified monoclonal antibody or pHSA was pre-incubated with 25 μl [125I] labelled HBV (100000 c.p.m.) for 1 h at room temperature. This was then added to the pHSA-coated wells for a further hour. The wells were washed five times with 1% BSA in PBS and counted.
Identification of polypeptide distribution of RF-HBs-1 epitope. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed using the discontinuous buffer system of Laemmli (1970). A 10% or 12.5% polyacrylamide separating gel with a 5% polyacrylamide stacking gel was used throughout. Gels were stained for protein using 0.25% Coomassie Brilliant Blue in 40% methanol, 10% acetic acid or silver stain (Bio-Rad). The mol. wt. of the polypeptides were estimated by reference to Bio-Rad low molecular weight standards run on every gel. Prior to SDS–PAGE samples were reduced by boiling for 5 min with 0.1 M-dithiothreitol and 1% SDS.

HBsAg was solubilized but not reduced by incubating for 2 h at 37 °C with 1% (w/v) SDS (Mishiu et al., 1980). Individual polypeptides were prepared by separating them on a 3 mm 12.5% polyacrylamide gel which was sliced into 0.5 cm strips and the polypeptides were eluted by mixing for 48 h with 0.125 M-Tris-HCl pH 6.8, 0.1% SDS buffer.

The polypeptides separated by SDS–PAGE were transferred to a nitrocellulose membrane using the Bio-Rad Trans-Blot cell. They were electrophoresed at 0.2 A for 16 h in 25 mM-sodium phosphate buffer pH 6.5. The blots were stained for protein with 0.5% amido black. The blots to be immunostained were blocked for 2 h in blocking buffer (Renast et al., 1979) (0.25% gelatine, 0.02% Triton X-100, 25 mM-sodium phosphate pH 6.8) and then incubated with 100 µl of ascites containing monoclonal antibody in 100 ml blocking buffer for 2 h. The blots were washed with blocking buffer for a further 2 h and incubated with 125I-labelled goat anti-mouse Ig (106 c.p.m. per lane in 50 ml of buffer). After washing in blocking buffer the blots were autoradiographed.

RESULTS

Competitive inhibition of RF-HBs-1 by naturally occurring antibodies to HBsAg

The detection limits of the competitive inhibition assay were estimated by the inhibition of binding of radiolabelled RF-HBs-1 to the HBsAg solid phase by unlabelled RF-HBs-1. The dose–response curves, using various amounts of HBsAg on the bead and 100000 c.p.m. of radiolabelled RF-HBs-1, are illustrated in Fig. 1. Over 90% inhibition of 125I-RF-HBs-1 was achieved with 1000 ng of unlabelled RF-HBs-1, and using beads coated with 0.5 µg/ml or 1.0 µg/ml HBsAg over 75% inhibition of binding occurred with 10 ng RF-HBs-1. Experiments were done using 50000 c.p.m. and 100000 c.p.m. radiolabelled RF-HBs-1 with similar dose–response curves but with lower maximum binding. Since the radioactivity added did not affect the percentage inhibitions achieved 100000 c.p.m. RF-HBs-1 was added throughout, and beads were coated with 1 µg/ml HBsAg.

In convalescent sera from 42 patients with acute type B hepatitis, there was a strong positive correlation (correlation coefficient r 0.626, P < 0.001) between the titre of antibody which bound to the RF-HBs-1 epitope as measured by percentage inhibition of binding and the total anti-HBs titre as measured by AUSAB (Fig. 2). Twenty-five normal human sera negative for HBs antibody allowed RF-HBs-1 to bind with a mean of 18257 (s.d. ± 2444) c.p.m. Among 18
patients' sera with an AUSAB titre of greater than 50 times the cut-off value, 15 gave a greater than 50% inhibition in the RF-HBs-1 inhibition assay. Of the 24 patients with a low AUSAB titre 21 had a correspondingly low RF-HBs-1 titre but three sera had a high titre of RF-HBs-1-inhibiting antibody.

**Inhibition of pHSA by RF-HBs-1**

The binding of pHSA to its receptor on HBV particles was inhibited to a similar degree by RF-HBs-1 and pHSA. RF-HBs-1 inhibited the binding of $^{125}$I-HBV particles by 78% at a concentration of 10 μg/ml, and at the same concentration pHSA gave 90% inhibition of binding (Fig. 3). Both RF-HBs-1 and pHSA gave 60% inhibition at a concentration of 0.01 μg/ml. Another IgG1 monoclonal antibody, RF-HBs-7, which binds to HBsAg but does not inhibit the binding of RF-HBs-1, did not inhibit the binding of $^{125}$I-HBV particles to pHSA (Fig. 3).
Hepatitis B virus neutralizing antibody

Fig. 5. Identification of RF-HBs-1 epitope on peptides derived by SDS solubilization of HBsAg. (a) Immunoblot of solubilized HBsAg with RF-HBs-1 (10% polyacrylamide gel). (b) The three polypeptides of mol. wt. 42000, 58000 and 75000 (left to right) bearing the RF-HBs-1 epitope were all shown to consist of the same two major polypeptides gp28 and p24 (12.5% polyacrylamide gel, silver-stained).

Analysis of RF-HBs-1 epitope

HBsAg, reduced with dithiothreitol and SDS and electrophoresed on an SDS–polyacrylamide gel which was then stained for protein with Coomassie Brilliant Blue, separated into a number of polypeptides (Fig. 4a). From the same patient, HBsAg treated with SDS only and electrophoresed, could only be visualized with silver stain. This revealed three bands of mol. wt. 75000, 58000 and a broader band of 41000 to 44000 (Fig. 4b). Reduced HBsAg, electrophoresed and transferred onto a nitrocellulose membrane and stained for protein with amido black gave the same polypeptide profile as on the gel; however RF-HBs-1 did not bind to these polypeptides. RF-HBs-1 did bind to HBsAg treated with SDS alone, electrophoresed and transferred onto a nitrocellulose membrane to give three bands of mol. wt. 75000, 58000 and 41000 to 44000 (Fig. 5a). The $^{125}$I-goat-anti-mouse used as the tracer did not bind to these bands when used alone.

These three bands were isolated from a preparative gel, reduced by treatment with dithiothreitol and SDS and separated by SDS–PAGE. All three bands separated into the two major polypeptides p24 and gp28 (Fig. 4b). Four faint bands of mol. wt. 66000, 57000, 46000 and 42000 were also visible in the reduced 58000 and 75000 polypeptides and a 50000 mol. wt. band in the reduced 42000 mol. wt. polypeptide.
Epidemiological studies (Melnick, 1981) and the use of the HBsAg vaccine (Szmuness et al., 1980) have demonstrated that the antibody response to HBsAg is important in protection against infection by HBV. In chimpanzees 10 μg of the murine monoclonal antibody RF-HBs-1 which recognizes the ‘a’ determinant of HBs antigen, has been shown to neutralize 1000 chimpanzee infectious doses of HBV of both ad and ay subtypes (Iwarson et al., 1985). We have demonstrated that antibodies which recognize the same epitope as RF-HBs-1 are present in the sera of the majority of patients recovering from an acute HBV infection and that this antibody response is closely related to the total anti-HBs titre. Antibody to this epitope forms a part of the natural immune response to the virus and this antibody would be expected to confer protection against re-infection.

RF-HBs-1, but not another IgG1 monoclonal antibody to HBsAg, RF-HBs-7, inhibits the binding of the virus to pHSA. The site of the pHSA receptor on the HBsAg polypeptides is the subject of controversy. Ionesco-Matiu et al. (1980) detected pHSA binding on the polypeptide p22 and p68 whereas Machida et al. (1983) found activity on the gp31 and gp35 polypeptides but not on p22 or p68. The latter group used a horse anti-HBs antibody, raised against native HBsAg particles to detect their binding and this would recognize predominantly conformational HBs gene-encoded epitopes and pre-S sequences present on gp31 and gp35 which have been shown not to be conformation-dependent (Neurath et al., 1984). Thus the pHSA-binding site may be present on all four polypeptides but was not recognized in the studies reported by Machida et al. (1983). Our data cannot directly confirm this but it does suggest that the RF-HBs-1 epitope, which is present on the S gene product, is topographically closely related to the pHSA receptor. Whether this property of RF-HBs-1 determines its virus-neutralizing ability remains to be determined.

The RF-HBs-1 epitope is conformationally dependent, being related to the linkage of two polypeptides by a disulphide bond. Under reducing conditions the disulphide bonds of HBsAg are broken and the antigen can be separated into several linear polypeptides of various molecular weights. Since RF-HBs-1 did not bind to these individual polypeptides, the epitope recognized by RF-HBs-1 is not present on the linear peptide sequence of the antigen. RF-HBs-1 did bind to three bands generated by solubilizing HBV particles with SDS and each of these bands when isolated and reduced consisted mainly of the two major polypeptides p24 and gp28. In each case bands of higher mol. wt. were detectable in very small amounts and these probably correspond to the high mol. wt. bands seen in all HBsAg preparations which are thought to be aggregates of the two major bands.

Systems for the detection of this virus-neutralizing antibody, which is present in the sera of patients who have recovered from an HBV infection, may have a use in the screening of potential recipients of future HBV vaccines.

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Hepatitis B virus neutralizing antibody


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