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Epitope Mapping of Neutralizing Monoclonal Antibodies against Duck Hepatitis B Virus

RAMSEY C. CHEUNG,[1,2] WILLIAM S. ROBINSON,[3] PATRICIA L. MARION,[3]* AND HARRY B. GREENBERG[1,2]

Division of Gastroenterology[1] and Division of Infectious Diseases,[3] Department of Medicine, Stanford University School of Medicine, Stanford, California 94305-5701, and Palo Alto Veterans Administration Medical Center, Palo Alto, California 94304[2]

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In this article we report the first topological mapping of neutralizing epitopes of a hepadnavirus. Duck hepatitis B virus is the only hepadnavirus that can replicate and spread from cell to cell in tissue culture. As a result, it is possible to study hepadnaviral neutralization in vitro with this system. To accomplish this goal, we produced a library of monoclonal antibodies against duck hepatitis B virus and identified 12 neutralizing monoclonal antibodies by using an in vitro neutralization assay. The characteristics of six of the neutralizing monoclonal antibodies were further studied by epitope mapping. From the results of competitive binding studies, three distinct neutralizing epitopes were identified on the pre-S polypeptides and one was identified on the S polypeptide. Our findings suggest that antibodies to both the pre-S and S gene products of duck hepatitis B virus can neutralize viral infection in vitro. The pre-S gene product is at least as important as the S gene product in eliciting neutralizing antibodies.

Hepatitis B is a major viral disease throughout the world and has been linked to primary hepatocellular carcinoma (1). It is conservatively estimated that about 5% of the global population, including 1.5 million people in United States, are chronic carriers of hepatitis B viral infection (34), which currently has no cure.

Despite the existence of effective vaccines (34), little is known about the neutralizing antigenic determinants or mechanisms of hepatitis B viral neutralization, mainly because of the lack of an in vitro propagation system. Two hepatitis B virus (HBV) polypeptide regions have been shown to elicit antibodies that protect chimpanzees from HBV infection, one encoded by the pre-S gene (8, 20) and the other encoded by the S gene (5, 24). An anti-idiotypic antibody vaccine (10) and a murine monoclonal antibody against a conformational determinant (9, 33) have also been shown to be protective in the chimpanzee model. In addition, antibodies against the e antigen and/or the core antigen of HBV were shown to protect the chimpanzees from HBV-associated hepatitis (18).

Duck hepatitis B virus (DHBV) is one of three or more animal viruses related to HBV, the hepadnavirus prototype. All of the hepadnaviruses have similar biological properties, ultrastructure, and genetic organization (7, 25, 31). The advantages of using ducks as an animal model for HBV include their ease of handling, ready availability, and 100% infection rate (14), but most important of all is the existence of an in vitro infection system (32). It is possible to study duck hepadnaviral neutralization quantitatively and in detail by using cell culture. Studies with a polyclonal rabbit serum against duck hepatitis B surface antigen (DHBsAg) demonstrated reduced infectivity of DHBV, whereas virus infectivity was not affected by polyclonal anti-core antibodies (32). Pugh et al. (23) later found that anti-pre-S antibodies but not anti-S antibodies were neutralizing in vitro.

We made monoclonal antibodies against purified DHBsAg particles to investigate the neutralizing epitopes on the surface polypeptides encoded by the S and pre-S regions. In this article we report the characterization of six neutralizing monoclonal antibodies directed at four different epitopes of DHBsAg.

MATERIALS AND METHODS

Isolation of DHBsAg particles. DHBsAg particles were isolated from serum with a high DHBV DNA titer (as shown by slot blot) by using CsCl gradient ultracentrifugation as previously described (15).

Hybridoma production. Two-month-old BALB/c mice (Institute for Medical Research, San Jose, Calif.) were immunized intraperitoneally with approximately 20 μg of purified DHBsAg in an equal volume of Freund complete adjuvant. The same dose mixed with incomplete Freund adjuvant was used approximately 3 weeks later. Mice were boosted one or two times more until a good immunologic response could be demonstrated by an enzyme-linked immunosorbent assay (ELISA) or by Western blotting (immunoblotting). Mice were sacrificed 3 or 4 days after a final intravenous inoculation. Spleen fusion, cloning, and ascites production were done as described previously (6) with the following modification: instead of NS1 cells, the fusion myeloma line was FOX cells (HyClone), and 7.5×10^{-5} M adenine–8×10^{-7} M aminopterin–1.6×10^{-5} M thymidine was used as the selection medium (29). Cells were plated out at a final density of 1.75×10^6 spleen cells per ml or 3.5×10^5 cells per well in 96-well plates (Costar). Hybridoma supernatants were screened by using the ELISA and an immunofluorescence assay. A single colony from each well was cloned twice by limiting dilution and was assumed to be monoclonal when the doubly cloned hybridoma produced a single subtype of immunoglobulin on isotyping (see below).

ELISA. Duck serum from congenitally infected ducks (DHBV DNA positive) or normal duck serum from uninfected ducks (DHBV DNA negative) was diluted in phosphate-buffered saline (PBS; Irvine Scientific), generally at a 1:500 dilution to coat 96-well microdilution plates (Immulon II; Dynatech Laboratories, Inc.). Plates were incubated overnight at 4°C. Hybridoma cell supernatants at 1:2 to 1:4 dilution in PBS were placed in each coated well for 2 h at

* Corresponding author.

37°C, washed three times with PBS–0.05% Tween 20 (Bio-Rad Laboratories), replaced with 1:1,000 peroxidase-conjugated rabbit anti-mouse immunoglobulin G (IgG; Sigma Chemical Co.), incubated for 1 h at 37°C, and washed five times with PBS–0.05% Tween 20. Plates were developed by using *o*-phenylene diamine (Sigma) as the substrate as described previously (27).

Immunofluorescence staining assay. Livers from congenitally DHBV-infected ducklings (or uninfected animals as control) were surgically removed. Small tissue sections were placed in Tissue-Tek (Miles Laboratories, Inc.) and frozen in liquid nitrogen. Frozen sections of the liver were prepared on glass slides, fixed in acetone, and stored at −20°C until use. Hybridoma supernatants were added to frozen liver sections on a slide after rehydration with PBS–2% bovine serum albumin (Sigma) and incubated in a moist chamber at 37°C for 30 min. The slides were washed with PBS for 10 min at room temperature. Fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG (Zymed) diluted in PBS–2% bovine serum albumin–2% seronegative normal duck serum was added and incubated for another 30 min at 37°C. After slides were washed in PBS for 10 min, they were mounted with 90% glycerol in PBS and 2.5% 1,4-diazabicyclo(2,2,2)octane and examined under a fluorescent microscope.

SDS-polyacrylamide gel electrophoresis and Western blotting. Duck serum with a high DHBV DNA titer was boiled for 3 min in a sample buffer (62.5 mM Tris base [pH 6.8], 3% sodium dodecyl sulfate [SDS], 10% glycerol, 5% 2-mercaptoethanol, 0.02% [wt/vol] bromophenol blue), loaded onto a 15% SDS–polyacrylamide gel, and electrophoresed with the buffer system described by Laemmli (11). After electrophoresis, the proteins were transferred to nitrocellulose paper (Schleicher & Schuell Co.) by using a Transblot apparatus (Bio-Rad) in a buffer of 25 mM Tris base, 192 mM glycine, 20% (vol/vol) absolute methanol, and 0.1% SDS. The efficiency of transfer was confirmed by the complete transfer of prestained low-molecular-weight protein standards (Bethesda Research Laboratories, Inc.) to the nitrocellulose paper. After transfer, the nitrocellulose paper was blocked with blotto mix (5% [wt/vol] nonfat dry milk in 10 mM Tris hydrochloride–155 mM NaCl). The blot was then mounted onto a Miniblotter (Immunetics) and incubated with diluted cell supernatants or ascites fluid at 37°C for 2 h. After the blot was washed with PBS–0.05% Tween 20, it was incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (Kirkegaard & Perry Laboratories) at a 1:1,000 dilution for 1 h at 37°C. After extensive washing with PBS–0.05% Tween 20, the blot was developed with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium phosphatase substrate system (Kirkegaard & Perry) until dark purple bands of the desired intensity appeared. The blot was washed with deionized water and air dried for a permanent record.

Hepatocyte culture. Hepatocytes were obtained by using a two-stage liver perfusion, described by Tuttleman et al. (32), of 10- to 14-day-old uninfected Pekin ducklings hatched at Stanford. Cells were seeded at approximately 3.6×10^5 cells per well in 24-well plate (Costar) in L-15 medium containing 5% fetal calf serum (FCS) and incubated at 37°C. The medium was changed every day for the first week and every other day for the second week.

In vitro neutralization assay. One day after plating, the monolayer was washed twice with L-15 medium–1% FCS, while DHBV from the yolk of an infected embryo (>3 ng of DHBV DNA per ml) at 1:2 dilution in PBS was preincubated with an equal volume of test hybridoma supernatant (undiluted) or ascites fluid (generally 1:100 in L-15 medium–1% FCS for screening) at 37°C for 1 h. After a further fivefold dilution of the mixture with L-15 medium–1% FCS, the mixture was added to the washed monolayer and incubated at 37°C for an additional 1 to 3 h. The inoculum was removed, and new L-15 medium–5% FCS was added. For the infected controls, inoculum virus was prepared as described above, with the exception that the ascites fluid and supernatants were replaced with L-15 medium–1% FCS and hybridoma cell culture medium, respectively. Cells were harvested from each well on day 14 postinfection in 0.1 ml of DNA extraction buffer (0.02 M Tris hydrochloride [pH 8.5], 0.01 M EDTA, 5 mM EGTA, 0.1 M NaCl). Proteinase K (2.5 μl; Sigma) at 20 mg/ml was added to each sample. Cells were freeze-thawed three times with an ethanol-dry ice bath and a 37°C water bath. DHBV DNA in the cell lysate was quantified by slot blotting (see below). The amount of DHBV DNA in the cell lysates 14 days postinfection was below the sensitivity of the slot blot assay (i.e., nondetectable) when wells were infected with the yolk at a 1:200 final dilution.

Viral DNA detection. We used a slot blot technique previously described (14) and modified for the cell lysates. Samples (100 μl) of cell lysates were denatured in 0.1 N NaOH for 5 min and neutralized with 135 μl of 7.5 M ammonium acetate and spotted. Samples were washed with 70% ethanol six times, 5 min each at room temperature, and soaked in 0.1 M triethanolamine (Sigma) with acetic anhydride for 10 min: Other steps, including hybridization with a ^{32}P-radiolabeled DHBV DNA dimer, were performed as described previously (14). Autoradiograms exposed to the hybridized blot on X-Omat AR film (Eastman Kodak Co.) were scanned with a scanning densitometer (Helena). The integrations associated with each slot were converted to nanograms of DHBV DNA per milliliter by using a standard curve derived from the known amount of blotted DHBV DNA.

Purification and biotinylation of monoclonal antibodies. Selected hybridomas were chosen for further studies. These hybridomas were cloned twice by limiting dilution and shown to contain a single type of antibody by isotyping with a mouse monoclonal sub-isotype kit (HyClone Laboratories). The monoclonal antibodies were amplified in mouse ascites fluids. Ascites fluids were collected from mice by paracentesis with a 20-gauge needle and processed as described previously (27). IgG was purified from the ascites fluid on a column of protein G (Pharmacia Fine Chemicals), and the purity of selected peak eluted fractions was checked by electrophoresis on a 15% SDS–polyacrylamide gel and staining with Coomassie blue. Purified IgG was quantified by using a Bio-Rad protein assay and biotinylated as described previously (27).

Competition binding studies. Competition binding studies compared the ability of the various unlabeled monoclonal antibodies to compete with biotinylated monoclonal antibodies for DHBV binding in an ELISA. The avidities of monoclonal antibodies were compared by titering similar known quantities of purified monoclonal antibodies (biotinylated and unlabeled) in an ELISA with DHBV as the solid phase. The studies were performed as described previously (27). The background for this assay (in the absence of biotinylated antibodies or DHBV precoat) was very low (optical density of <0.05; data not shown).

The level of competition by unlabeled heterologous monoclonal antibodies was compared with that provided by the labeled counterpart (homologous) of the biotinylated antibodies. Competition was defined as significant if the concentration of an unlabeled heterologous antibody necessary to achieve 70% or greater inhibition was less than 2.5 times the

concentration of the unlabeled homolog of the biotinylated antibody necessary to produce a similar degree of binding inhibition. Monoclonal antibodies that competed significantly were presumably directed at the same or closely related epitopes on the virus.

RESULTS

Hybridoma screening. Hybridoma cell supernatants were screened for antibodies against DHBV by both the ELISA and the immunofluorescence assay. Out of a total of 100 hybridoma cell supernatants from the 96-well plates that were screened, initially 84 were considered ELISA positive with positive/negative ratios of >5. The negative control wells were precoated with DHBV-DNA-negative duck serum instead of virus-positive serum. After cells were transferred to 24 well plates, only 43 hybridoma cell supernatants remained positive by the ELISA. The remarkably high incidence of DHBV-specific hybridoma supernatants may be due to our high plating density of spleen cells (3.5×10^5 per well).

The ELISA was originally developed by coating the wells with DHBsAg purified from DHBV-positive serum by ultracentrifugation. However, we were able to simplify the assay by using DHBV-positive serum to coat the wells without sacrificing any sensitivity and specificity as long as the appropriate control, i.e., DHBV-negative serum, was used (data not shown). Furthermore, with unpurified DHBV-positive serum the assay has the potential to detect additional epitopes that may be reduced in amount during the CsCl purification steps. The ELISA was more sensitive than the immunofluorescence assay; only 49 of the 84 ELISA-positive cell supernatants were also positive by fluorescent staining. The specimens that were only positive in the ELISA were unlikely to be false-positives, since they were all screened simultaneously with a negative control, and a high positive/negative ratio cutoff was chosen to increase the specificity of the assay. However, eight supernatants were positive by the fluorescence assay only. They were only weakly fluorescent positive and scored trace to 1+, except one that was 3+ on immunostaining with a positive/negative of 2. This monoclonal antibody was directed at pre-S on Western blot and was nonneutralizing. The intensity of the fluorescent staining was graded as trace, 1+, 2+, and 3+. Trace meant the intensity of fluorescence was barely detectable above the background staining of uninfected control animals, and 3+ indicated the strongest intensity. Only the cytoplasm was stained at the low magnification for screening.

Western blot analysis. The specificities of the monoclonal antibody cell supernatants were characterized at the viral protein level by using a standard Western blot technique. Out of 59 supernatants that were tested, 34 were directed at the pre-S polypeptide, as shown by binding to single (occasionally double and rarely three) band(s) of 34 to 37 kilodaltons (kDa) (Fig. 1). The relative sizes of these species were consistent with the published data on the large envelope protein of DHBV (23, 26). Eight monoclonal antibodies were directed to the S polypeptide as shown by detection of a 17-kDa band in addition to the 34- to 37-kDa bands. Of the 59 monoclonal antibodies, 17 were nonreactive on Western blots. Of the 40 supernatants that were both ELISA and fluorescence positive, 27 were directed at the pre-S polypeptide, 6 were directed at the S polypeptide, and only 7 were nonreactive on the Western blots. Those that were nonreactive on Western blots were either directed to epitopes that were not present on the blot (i.e., conformational epitopes) or not detected because of being below the sensitivity of the assay (cell supernatants contain only small amount of antibodies [<100 μg/ml]). Since these seven cell supernatants were positive by both screening assays, they were most likely directed at conformational epitopes. Western blot detection was found to be specific since all the controls including the tissue culture medium alone, cell supernatant from a monoclonal antibody against rotavirus, and cell supernatant from the fusion murine myeloma cell line were nonreactive (data not shown). Selected cell supernatants were tested by Western blotting with DHBV DNA negative duck serum and they were also nonreactive.

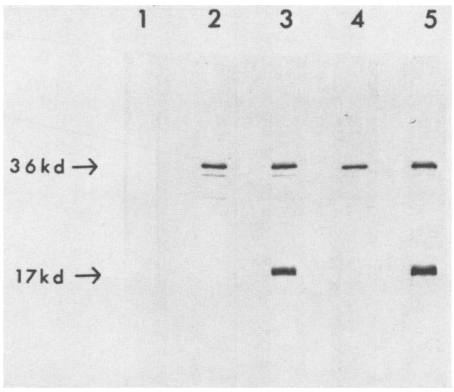

FIG. 1. Western blots of neutralizing monoclonal antibodies against DHBV polypeptides separated by electrophoresis on a 15% SDS–polyacrylamide gel with DHBV-negative control serum in lane 1 and DHBV-positive sera in lanes 2 through 5 (serum from duck A in lanes 2 and 3, serum from duck B in 4 and 5). After incubation with anti-pre-S antibody 1E2 for lanes 1, 2, and 4 and anti-S antibody 1B10 in lanes 3 and 5, the blot was developed by using an alkaline phosphatase-conjugated system (see Materials and Methods for details).

In vitro neutralization. Cell supernatants were initially screened at a final dilution of 1:10 with the viral inoculum at a 1:20 dilution. As mentioned above, viral replication was undetectable when the viral inoculum was used at a 1:200 dilution. Ascites fluid were tested at a 1:1,000 final dilution with the same amount of viral inoculum. A monoclonal antibody that produced a 50% or greater reduction in detectable DHBV DNA at 14 days incubation was considered to have neutralizing activity. Attempts to measure neutralization earlier after infection (7 and 10 days postinfection) were unsatisfactory, because the level of detectable viral DNA was too low (data not shown). Using such a system to screen 45 hybridoma cell supernatants and ascites fluids, we found that 12 had neutralizing activity. Eleven were directed at the pre-S polypeptide on Western blots; one was directed at the S protein. They were all positive in both the ELISA and the immunofluorescent assay. Six had high neutralizing activities and were chosen for further studies, whereas the other six with only low levels of neutralizing activities were not examined further.

To study the effect of monoclonal antibody concentration on the neutralizing activity, selected monoclonal antibodies that were cloned twice and amplified as ascites fluid were tested at four different dilutions, 1:100 to 1:6,400 (Fig. 2). Monoclonal antibodies showing neutralizing activity by initial screening exhibited dilution-dependent neutralizing activity. This was not observed with the ascites fluid containing non-neutralizing monoclonal antibodies. Several

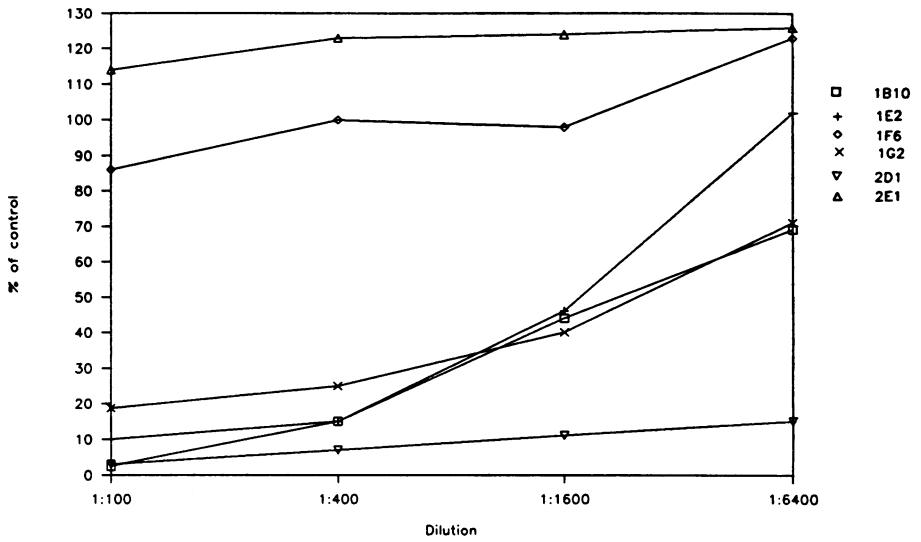

FIG. 2. Effect of dilution of monoclonal antibody ascites fluid on in vitro neutralizing activity. Neutralizing activity was assayed by using an in vitro infection system as described in Materials and Methods. Viral replication was quantified by measuring the amount of intracellular DHBV DNA in the total cell lysates 14 days postinfection. This is expressed on the *y* axis as percentage of infected controls. The *x* axis represents dilution of monoclonal antibody ascites fluid in L-15 containing 1% FCS. 2E1 and 1F6 are non-neutralizing anti-S and anti-pre-S monoclonal antibodies, respectively.

monoclonal antibodies that were considered non-neutralizing by the initial screening with the cell supernatants showed neutralizing activity with the ascites fluid, probably because the antibody concentrations in the cell supernatants were too low to achieve significant neutralization. None of the monoclonal antibody ascites that was non-neutralizing at 1:1,000 showed neutralizing activity even at a low dilution such as 1:100, indicating that the initial screening test was sensitive.

Epitope mapping of neutralizing monoclonal antibodies. Six neutralizing monoclonal antibodies were chosen for epitope mapping, including the only anti-S neutralizing monoclonal antibody (1B10). Each antibody was cloned twice and was determined to have a single subclass. The relative avidities to DHBV were determined by titration in an ELISA. All anti-pre-S neutralizing monoclonal antibodies demonstrated similar binding curves, suggesting similar avidities (28; data not shown). Representative results of the competitive binding studies are shown in Fig. 3 and 4 and summarized in Fig. 5 and Table 1. All antibodies significantly inhibited viral binding of the labeled homologous counterpart. Three of the five anti-pre-S monoclonal antibodies (namely, 1E2, 1H5,

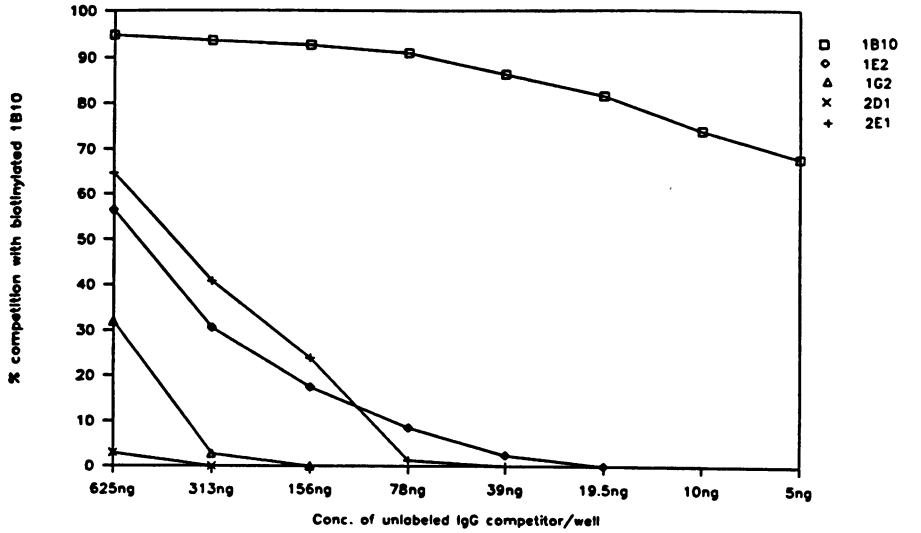

FIG. 3. Competitive binding ELISA of several monoclonal antibodies in competition with biotin-labeled 1B10, a neutralizing anti-S antibody. 2E1 is a non-neutralizing monoclonal antibody against the S protein, and the rest are neutralizing anti-pre-S antibodies. A fixed concentration of biotin-labeled 1B10 was mixed with decreasing amounts of unlabeled purified IgG from competing monoclonal antibodies. The assay was performed as described in the text. Percentage competition was defined as [1 − (OD_{490} with unlabeled second antibody/OD_{490} with biotinylated antibody only)] × 100, where OD_{490} is the optical density at 490 nm. Competition was defined as significant if the quantity of unlabeled heterologous competitor to produce 70% or greater inhibition was less than 2.5 times the quantity of homologous competitor required to produce same degree of inhibition. No competitor other than homologous 1B10 produced significant competition.

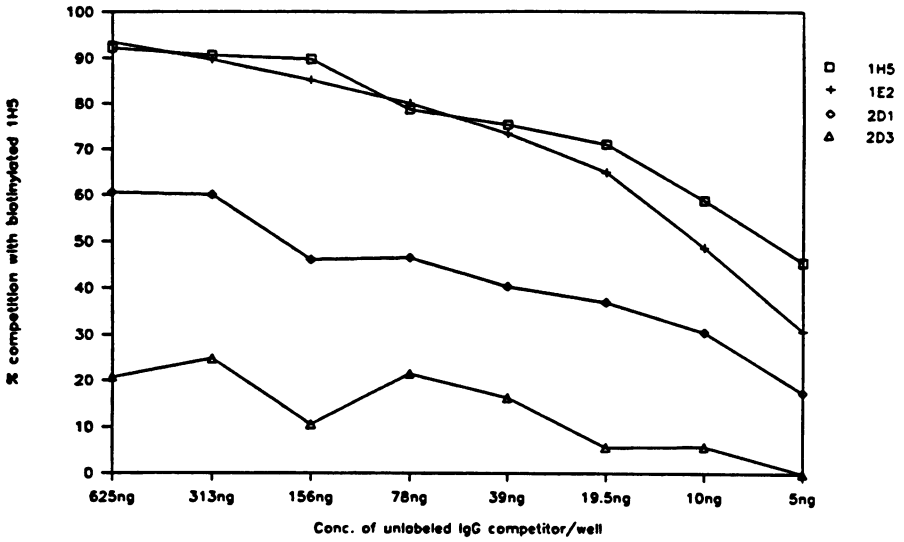

FIG. 4. Competitive binding ELISA of several monoclonal antibodies in competition with biotinylated 1H5, a neutralizing anti-preS antibody. See the legend to Fig. 3 for details. 1E2 showed significant competition with biotinylated 1H5, and hence 1E2 is considered to be directed at the same or closely related epitope as 1H5.

and 2C11) competed significantly with each other and also showed reciprocal inhibition (Fig. 4). They did not compete, however, with the other anti-pre-S neutralizing monoclonal antibodies such as 1G2 and 2D1 or with the anti-S neutralizing monoclonal antibody 1B10. These three neutralizing monoclonal antibodies, 1E2, 1H5, and 2C11, were considered to be directed at the same or closely related epitopes. The other two anti-pre-S neutralizing monoclonal antibodies, 1G2 and 2D1, did not compete significantly with any other neutralizing monoclonal antibodies with the exception of the homologous unlabeled counterpart. Similar findings were also observed with 1B10. In addition, 1B10 did not compete significantly with 2E1, a non-neutralizing anti-S monoclonal antibody. Antibodies that mutually inhibited each other's binding to the virus were assigned to the same epitope (Table 1). The competitive inhibition analysis of these first six neutralizing monoclonal antibodies demonstrated that there were at least four neutralizing epitopes on the DHBV surface antigens. Only one neutralizing epitope was identified on the S polypeptide.

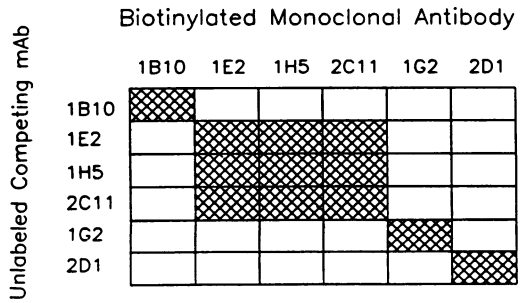

FIG. 5. Summary of competitive binding assays of biotinylated neutralizing monoclonal antibodies with purified unlabeled antibodies. The cross-hatched rectangles represent significant inhibition by the competitor as defined in Fig. 3; open rectangles indicate no significant competition. All monoclonal antibodies were neutralizing in vitro and purified from mouse ascites fluid of doubly cloned cells and were shown to contain a single isotype.

DISCUSSION

To our knowledge, this is the first report on the topological mapping of neutralizing epitopes of a hepadnavirus. The mapping of DHBsAg neutralizing epitopes was made possible through development of an in vitro infection system for DHBV (32). Among the mammalian hepadnaviruses, only limited information exists for neutralizing epitopes of HBV and antigenic sites on the woodchuck hepatitis virus (2). At least one neutralizing murine monoclonal antibody against HBsAg of the IgG1κ subclass has been shown to be protective in chimpanzees (9), and the specificity of this monoclonal antibody has been determined to be a conformational epitope that was part of or near the polymerized human serum albumin-binding site, which is encoded by the pre-S2 sequence (33). The pre-S region is subdivided into the pre-S1 region, which codes for 108 to 119 amino acids (depending on the subtype), and the pre-S2 region for 55 amino acids on the carboxyl end (31). Antibodies to at least two regions on the surface of HBV (amino acid sequences pre-S 120 through 151 and S 110 through 137) protect chimpanzees from HBV infection (5, 8, 20, 24). The fine specificity of the idiotype paratope in a protective anti-idiotype vaccine was found to center around amino acid sequences S 117 through 137 of the HBV surface antigen (4). This coincided with the findings of

TABLE 1. Characteristics of neutralizing monoclonal antibodies to DHBV

Monoclonal antibody	IgG subclass[a]	Specificity	
		Viral protein[b]	Epitope[c]
1B10	G2a	S	I
1E2	G1	Pre-S	II
1H5	G1	Pre-S	II
2C11	G1	Pre-S	II
1G2	G2b	Pre-S	III
2D1	G1	Pre-S	IV

[a] The IgG subclasses were determined by using a mouse monoclonal subclass isotyping kit (HyClone Laboratories).
[b] As determined by Western blotting against DHBV.
[c] As determined by competitive binding studies (Fig. 4).

Gerin et al. (5) and Purcell and Gerin (24). However, the amino acid sequence did not include that of a putative HBV cell receptor-binding site located in the pre-S region, pre-S 21 through 47 (21); therefore the role of virus cell receptor-binding site in neutralization is unclear. Even though the S gene product is highly conserved among the hepadnaviruses, the S amino acid sequence that was found to be important in HBV neutralization is missing in the DHBV, and the pre-S region is highly variable between HBV and DHBV and among subtypes of both (12). Therefore, DHBV neutralizing monoclonal antibodies are most likely directed at amino acid sequences distinct from those known in HBV; some may even encode a DHBV cell receptor-binding site(s). Experiments in our laboratory are in progress to determine the amino acid sequences to which these neutralizing monoclonal antibodies are directed. Since all of the neutralizing monoclonal antibodies bind in Western blots, it should be possible to locate the amino acid sequences to which the antibodies are directed by either bacterially expressed gene products or synthetic peptides.

Based on amino acid hydrophilicities and three-dimensional computer analysis, the pre-S sequence in HBV was more antigenic (and probably more immunogenic) than the S protein in HBV (19). We isolated more anti-pre-S than anti-S hybridomas, consistent with this prediction. Schlicht et al. (26) also found that the major immunologic response in immunizing rabbits with undenatured DHBsAg particles, consisting of S and pre-S antigen, was directed against pre-S determinants.

DHBV has six in-frame start codons in the pre-S region, encoding polypeptides of approximately 37, 36, 35, 34, 31, and 28 kDa. Currently it is not known how many of these codons are functional. Schlicht et al. (26) found a major pre-S protein of 35 kDa and a minor species of 37 kDa appearing in some sera. N-terminal amino acid sequence analysis of the 35-kDa pre-S protein was unsuccessful. Another laboratory identified a 36-kDa protein as the major pre-S species, with a less prominent 34-kDa protein occasionally observed (23). Western blots of some duck sera with anti-pre-S monoclonal antibodies demonstrate up to three protein bands of 34 to 37 kDa, with the 36-kDa protein being the dominant band (Fig. 1). The 34-kDa band was seen only occasionally. The minor bands of 34 and 37 kDa seen on Western blots represent either degradation products of the pre-S protein or, more likely, pre-S proteins with alternative start codons. Similar to observations in other laboratories, the minor protein bands were seen occasionally and with only certain sera, suggesting that start-codon utilization may depend on the stage of viral replication in the ducks. It is not clear at the moment what function might be served by having multiple pre-S proteins produced with several distinct start codons, nor is it known whether each species is found on the actual virion.

All competitive binding studies have to be interpreted in the context of relative avidity. All neutralizing monoclonal antibodies reported in this study showed similar avidities. Three of the four neutralizing epitopes were determined to be in the pre-S region of DHBV, suggesting that the pre-S region is probably as important as, if not more important than, the S region in terms of viral neutralization. This has important clinical implications, since both currently licensed vaccines contain none or a minimal amount of pre-S polypeptide. Although S protein alone has been shown to elicit protective immunity in a high proportion of vaccinees (34), there are additional advantages to using or incorporating the HBV pre-S gene product into future generations of hepatitis vaccine. Milich et al. (16) demonstrated in mice that a pre-S1-specific T-cell response could bypass the nonresponsiveness to the pre-S2 and S regions of HBsAg. Similarly, the pre-S2 sequence contains immunodominant epitopes and could circumvent the nonresponsiveness to S polypeptide through pre-S2-specific T-cell helper function (17). Furthermore, antibodies to synthetic peptides of the immunodominant epitopes in the pre-S region were subtype independent (22).

Our results, showing that antibodies to both pre-S and S polypeptides of DHBV have neutralizing activities, are consistent with what has been reported. Pugh et al. (23) found that polyclonal rabbit antibodies to a fusion protein containing 86 amino acids of the DHBV pre-S region decreased infectivity in a similar tissue culture infection system. Antibodies against a gel-eluted 17-kDa S protein did not affect infectivity. Pugh et al. suspected that the low titer of antibodies might account for the lack of effect on viral infectivity. Our studies showed that certain monoclonal antibodies were non-neutralizing in the cell supernatant but had neutralizing activity with ascites fluid, confirming that titer could be important. Earlier studies by Tuttleman et al. (32) with the same infection system showed that polyclonal rabbit antisera against undenatured purified DHBsAg particles were virus neutralizing. The polyclonal antibodies most likely have both anti-S and anti-pre-S antibodies but more of the latter, since Schlicht et al. (26) have already shown that the major immune response after immunizing rabbit with undenatured S particles was directed against pre-S determinants.

The specific mechanisms of hepadnaviral neutralization have yet to be determined. In the poliovirus system, which has been extensively studied, there are multiple neutralization mechanisms; distinct neutralizing monoclonal antibodies neutralize by different mechanisms (3, 13). Aggregation appears to be important in neutralization of poliovirus (30). This may be one of the mechanisms in DHBV neutralization as well, but it did not appear to be the sole mechanism since we also have many other monoclonal antibodies against DHBsAg that theoretically could aggregate the virus but were non-neutralizing. We have described six neutralizing monoclonal antibodies directed at four different epitopes on the DHBV surface. These most likely neutralize viral infection via more than one mechanism. It will be interesting to determine whether protection in vivo is associated with specific neutralization mechanisms or neutralizing antibodies to specific epitopes.

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