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Virus-Neutralizing Monoclonal Antibody to a Conserved Epitope on the Duck Hepatitis B Virus Pre-S Protein

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In this study we used duck hepatitis B virus (DHBV)-infected Pekin ducks and heron hepatitis B virus (HHBV)-infected heron tissue to search for epitopes responsible for virus neutralization on pre-S proteins. Monoclonal antibodies were produced by immunizing mice with purified DHBV particles. Of 10 anti-DHBV specific hybridomas obtained, 1 was selected for this study. This monoclonal antibody recognized in both DHBV-infected livers and viremic sera a major (36-kilodalton) protein and several minor pre-S proteins in all seven virus strains used. In contrast, pre-S proteins of HHBV-infected tissue or viremic sera did not react. Thus, the monoclonal antibody recognizes a highly conserved DHBV pre-S epitope. For mapping of the epitope, polypeptides from different regions of the DHBV pre-S/S gene were expressed in *Escherichia coli* and used as the substrate for immunoblotting. The epitope was delimited to a sequence of approximately 23 amino acids within the pre-S region, which is highly conserved in four cloned DHBV isolates and coincides with the main antigenic domain as predicted by computer algorithms. In in vitro neutralization assays performed with primary duck hepatocyte cultures, the antibody reduced DHBV infectivity by approximately 75%. These data demonstrate a conserved epitope of the DHBV pre-S protein which is located on the surface of the viral envelope and is recognized by virus-neutralizing antibodies.

Human hepatitis B virus (HBV), the prototype member of the hepadnavirus family (23, 55, 61), is a small enveloped DNA virus which induces acute and chronic liver diseases in humans. Related viruses have been isolated from woodchucks (63), ground squirrels (40), ducks (DHBV) (41, 69), and herons (HHBV) (58). Three HBV envelope proteins, designed pre-S1 (P39/GP42), pre-S2 (GP33/GP36), and S (P25/GP27), have been identified and are encoded within the pre-S/S open reading frame of the viral genome (43). They are synthesized by initiation of translation at three distinct AUG codons and are coterminal. They carry epitopes involved in immune defense mechanisms (10, 21, 24, 28, 35, 42, 46) and play a role in virus cell interaction (12, 45, 46, 49, 53). During infection, antibodies to pre-S proteins develop early, whereas antibodies to S are late markers and signal virus elimination (24, 28, 42, 46). The protective character of antibodies to S protein is well documented, and epitopes responsible for virus neutralization have been mapped (10, 21). Since there is no established cell line which can be infected with HBV and since chimpanzees are the only other animals susceptible to HBV, the role of pre-S antibodies in virus neutralization is less well studied. Immunization of chimpanzees with pre-S-specific synthetic peptides or HBV preincubated with antibodies to a synthetic peptide was shown to be protective (15, 27, 44). Whether the neutralizing activity in these experiments was due solely to neutralizing antibodies or also to cellular immune response mechanisms is not clear. To circumvent the problems associated with the functional analysis of pre-S proteins of HBV in immune defense mechanisms, we used DHBV as a model.

Ducks infected with DHBV represent the most convenient animal system in which the role of envelope proteins can be studied both in vitro and in vivo. Viruses with defined nucleotide sequences can be produced by transfection of animals with cloned viral DNA (59), which facilitates the interpretation of neutralization studies. Primary hepatocyte cultures can be reproducibly established and are infectable with virus (64), permitting an investigation into the neutralizing activity of antibodies independent of the effects of other immune defense mechanisms.

HBV and DHBV (or HHBV) are the least closely related hepadnaviruses, and this has to be taken into account when DHBV-infected animals or tissue culture cells are used. The major envelope protein of DHBV is only 17 kilodaltons (kDa), in contrast to that of HBV (25 kDa), and antibodies to human S protein do not cross-react with the DHBV S protein (DHBs) (17, 36, 40, 58), presumably because the corresponding antigenic region is lacking in the DHBV S gene (60). Also in contrast to HBV, so far only one major pre-S protein (of 36 kDa) has been consistently identified in sera and livers of infected ducks by several laboratories (17, 39, 50, 54, 58). More recently, a second major putative DHBV pre-S protein (of 28 kDa) has been discovered in infected liver and serum (68). The synthetic pathway for this protein and its function are not yet clear. In this paper, we describe a DHBV-specific monoclonal antibody with neutralizing activity which binds to a conserved epitope present on the major 36-kDa protein and several smaller DHBV pre-S proteins located on the surface of viral particles.

MATERIALS AND METHODS

Viruses. Several DHBV isolates originating in different geographic areas were experimentally transmitted to domestic French Pekin ducklings by inoculation with DHBV-positive sera. The viruses used for infection were from (i) a domestic French Pekin duck (DHBV Fp) (5), (ii) a French

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wild mallard (DHBV Fm) (6), (iii) an American wild mallard (DHBV Am) (32), (iv) two Chinese brown ducks (DHBV C and DHBV 26), (v) a goose (DHBV 1), and (vi) a German domestic Pekin duck (DHBV 3) (60). The viremic sera and infected liver tissues containing DHBV 26 and DHBV 3 were from ducks previously transfected with the corresponding cloned DHBV DNA of known sequence (59; unpublished data). From both the DHBV 1- and DHBV 26-containing sera, one DHBV DNA molecule was cloned and sequenced (R. Sprengel and H. Will, unpublished data). For immunoblot analysis, virus particles were concentrated from the serum by centrifugation through a 10 to 20% sucrose gradient as described previously (19). Heron serum containing high titers of HHBV from which a HHBV genome has been cloned and sequenced, and the corresponding infected liver originated from northern Germany (58).

Hybridoma production. Hybrid cell lines producing antibodies to the DHBV particles were obtained following fusion of mouse myeloma Sp2/0 Ag 14 cells (56) with mouse spleen cells by the method of Köhler and Milstein (29). The spleen cell donor was a BALB/c mouse (Charles River Breeding Laboratories, Saint-Aubin-les-Elbeuf, France) immunized twice at 2-week intervals by intraperitoneal injection of purified DHBV Fm particles (50 μg of protein in incomplete Freund adjuvant). For mouse immunisation, DHBV particles were purified from the serum of a highly viremic French mallard duckling (DHBV Fm) by CsCl gradient centrifugation as previously described (19). Mouse cells were fused 3 days after an intravenous booster injection of antigen (50 μg of protein in phosphate-buffered saline [PBS] solution). Hybridoma supernatants were screened by the enzyme-linked immunosorbent assay (ELISA) and an immunofluorescence assay. The antibody-producing hybridoma cells were cloned by limiting dilutions at least twice, and cells from the last cloning were injected into BALB/c mice pretreated by an intraperitoneal injection of Pristane (Aldrich Chemie, Steinheim, Federal Republic of Germany) to make ascites fluid. The class and subclass of monoclonal antibodies were determined by using antibody capture on antigen-coated plates with a streptavidin-biotin system/MonoAb SP Mouse kit (Zymed Laboratories, Inc., South San Francisco, Calif.). For production of a monoclonal antibody to the amino-terminal region of the polymerase protein of bacteriophage MS2, an MS2 fusion protein was purified and used for immunization.

ELISA. Direct ELISA was carried out by the method of Engvall and Perlmann (16). Optimal dilutions of antigens (0.25 to 2.5 μg of purified virus per 100 μl) were determined by checkerboard titration (66) with a polyclonal antiserum from a mouse previously immunized with DHBV. As a negative control, proteins concentrated from a DHBV-negative duck serum were used. The antigen dilution in coating buffer (100 μl in 0.1 M carbonate buffer [pH 9.6]) was coated overnight at room temperature into 96-well microtiter plates (Falcon Pro-bind; Becton Dickinson Labware, Oxnard, Calif.). The plates were then washed three times in PBS buffer containing 0.05% Tween 20 (PBS-Tween), and then 100 μl of hybridoma cell supernatants diluted in PBS-Tween was incubated for 2 h at 37°C. Alkaline phosphatase-conjugated goat anti-mouse total immunoglobulins (Zymed Laboratories, Inc., South San Francisco, Calif.) diluted 1:750 were added to each well and incubated for 1 h at 37°C. After the wells had been washed with PBS-Tween, substrate solution [1 mg of bis(*p*-nitrophenyl)phosphate (Sigma Chemical Co., St. Louis, Mo.) in 10% diethanolamine buffer (pH 9.8) per ml] was added. The optical density was read at 405 nm on a Titertek Multiskan (Flow Laboratories, Inc., McLean, Va.).

Immunofluorescence. Indirect immunofluorescence studies were carried out as described previously (65). Briefly, frozen liver specimens from DHBV-infected ducks and uninfected controls were cut on a cryomicrotome. The 4-μm-thick liver sections obtained were incubated with hybridoma supernatants, washed three times in PBS, and then incubated with fluorescein isothiocyanate-labeled anti-mouse immunoglobulins (Nordic). Finally, the slides were washed with PBS, mounted in 50% glycerol–PBS, and examined by microscopy.

Immunoblotting procedure. Duck liver homogenates prepared as described previously (68) or concentrated DHBV particles were heat denatured in 1% sodium dodecyl sulfate (SDS)–100 mM dithiothreitol in 100 mM Tris hydrochloride (pH 6.8). Proteins were separated on SDS–12.5% polyacrylamide gels by the method of Laemmli (31) and electrotransferred onto nitrocellulose filters (BA85; Schleicher & Schuell, Dassel, Federal Republic of Germany) (30). The filters were saturated with 10% dry milk in PBS-0.05% Tween 20 for 2 h and then incubated with diluted cell supernatants or ascites fluid for at least 12 h. After five washes in PBS-Tween, the blots were incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-mouse immunoglobulins (TAGO; Biosoft, Cambridge, United Kingdom) at a 1:1,000 dilution. After extensive washing with PBS-Tween, the filter was developed with diaminobenzidine (Sigma) until visible bands appeared. For the immunoblots with the recombinant viral proteins, a previously described procedure was used (22).

In vitro neutralization assay on primary duck hepatocyte cultures. Duck hepatocytes from a DHBV-negative animal were obtained by two-step collagenase perfusion of the liver (18). Briefly, cells were suspended in medium composed of 75% minimum essential medium and 25% medium 199 supplemented with 0.1% bovine serum albumin, 5 mg of bovine insulin per ml, and 7×10^{-5} M hydrocortisone hemisuccinate. Cells (7×10^6 per 75-cm^2 Falcon flask) were seeded and maintained at 37°C. The in vitro neutralization assay was performed 1 day after plating. Two multiplicities of infection (MOI; ratio of viral particles to hepatocytes of 280:1 and 28:1, respectively) were tested. Sera from DHBV-infected ducklings (diluted to 2×10^9 and 2×10^8 virus particles per ml [final dilution]) were preincubated for 1.5 h at 37°C with 1:200-diluted ascites fluid from anti-DHBV secreting clones or from mouse myeloma cells SP2/0, respectively, and then used to infect cells (six flasks per MOI). As a positive control of infection, the same MOI was used without previous incubation with monoclonal antibody. The inoculum was in contact with the cells for 1 h at room temperature, and then it was removed, cells were washed with PBS, and 10 ml of fresh medium was added. The medium was changed every 2 days and saved for further studies.

Detection of viral DNA. The DHBV DNA level both in hepatocytes and in the corresponding supernatants was measured. DNA was detected by dot blot hybridization of culture medium spotted onto nitrocellulose by using a HybriDot apparatus (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). After denaturation and neutralization of the fixed DNA (5), filters were hybridized as described below. Total DNA of hepatocytes was prepared from cells harvested on day 9 postinfection as previously described (18). Briefly, after incubation of the cells suspended in TEN solution (10 mM Tris hydrochloride [pH 7.4], 1 mM EDTA, 100 mM NaCl) with proteinase K (200 μg/ml) in the presence

of 0.1% SDS, proteins were removed by extraction with phenol-chloroform and nucleic acids were precipitated with ethanol. After gel electrophoresis of 10 μg of total nucleic acid, DNA was denatured and transferred to nitrocellulose by the procedure of Southern (57) as modified by Wahl et al. (67). DHBV DNA of infected cells and virus particles or cell culture supernatants was quantified by dot spot hybridization and counting of the radioactivity.

For probe preparation, genome-length DHBV DNA was excised from a plasmid containing cloned DHBV 16 DNA (a gift from W. Mason, Fox Chase Cancer Center, Philadelphia, Pa.), purified from low-melting-point agarose gel, and labeled by nick translation (52) to a specific activity of 0.8×10^8 cpm/μg in a reaction containing [α-^{32}P]dCTP (3,000 Ci/mmol). Hybridization was performed at 42°C as described previously (38).

Computer analysis of the protein sequence. DNA sequences from several cloned DHBV DNAs (36, 60; Sprengel and Will, unpublished data) and one HHBV DNA (58) were compared. Secondary structures of proteins were predicted by using the computer package of Deléage et al. (7, 8) based on the prediction methods of Chou and Fasman (4), Garnier et al. (20), and Levin et al. (33). The solvent accessibility, hydrophilicity, and antigenicity were predicted (13, 25, 47). The method for predicting the antigenicity of the protein (47) is based on factors such as flexibility and hydrophilicities. The most probable antigenic sites were predicted (13, 14, 25, 47).

Expression of DHBV pre-S proteins in *Escherichia coli*. For expression of DHBV pre-S proteins in *E. coli*, a full-length DHBV genome was isolated from plasmid DHBV16-t-27 (59) by *Bam*HI digestion and cloned into a procaryotic expression vector, pEx34a (62), previously linearized with *Bgl*II. Deletion mutants of the pre-S/S open reading frame were created by linearization of pEx34aDHBV16 at the unique *Bgl*II site in DHBV 16. The DNA was incubated with nuclease BAL 31 at 32°C in 0.6 M NaCl–20 mM Tris hydrochloride (pH 8.0)–12 mM $MgCl_2$–12 mM $CaCl_2$–1 mM EDTA, and the reaction was stopped with the same volume of 0.1 M ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA). To remove all DHBV sequences between the *Pst*I site in the polylinker and the *Bgl*II site in DHBV, we digested the mixture with *Pst*I. The resulting DNA fragments were blunt ended with the exonuclease activity of Klenow polymerase, electroeluted from a 1% agarose gel, ligated, and transformed into *E. coli* 2136 by established procedures (38). Plasmid DNA was isolated from colonies which expressed hybrid proteins visible by Coomassie blue staining after separation by SDS-polyacrylamide gel electrophoresis or visible by immunoblotting only, and the vector/DHBV-DNA junction was determined by dideoxy sequencing with a synthetic oligonucleotide as the primer (22). For immunoblotting, protein extracts of *E. coli* cells were prepared as described previously (22).

RESULTS

Generation of anti-DHBV pre-S monoclonal antibodies. Ten anti-DHBV-positive hybridomas as determined by ELISA were obtained in the fusion of SP2/0 with immune spleen cells of the mouse immunized with DHBV particles. One of six monoclonal antibodies which gave a similar pattern in immunoblotting (designated SD20) was selected for this study. This clone secreted immunoglobulin G1 lambda class and reacted specifically with DHBV particles in ELISA (data not shown). Furthermore, in immunofluorescence assays with liver tissue sections it recognized only DHBV-infected liver, and recognition could be inhibited by prior incubation of the monoclonal antibody with purified DHBV particles but not with control proteins. The fluorescent staining was cytoplasmic (data not shown). When purified DHBV particles were analyzed by immunoblotting, SD20 always revealed a major protein of 36 kDa (P36) and usually also several minor bands (Fig. 1A). Occasionally, a protein of 28 kDa was also reactive (Fig. 1A). The sizes of all immunoreactive proteins are similar to those described by DHBV pre-S proteins (17, 39, 50, 54, 58). This strongly suggests that SD20 recognizes an epitope of pre-S proteins. This interpretation is also consistent with the cytoplasmic localization of the antigen as independently shown by immunohistology with a polyclonal antibody to a pre-S protein expressed in *E. coli* (data not shown).

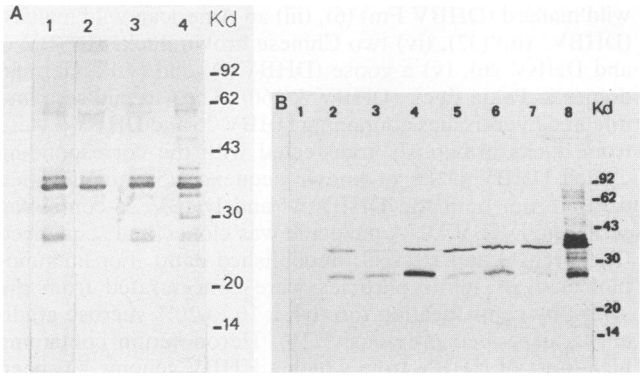

FIG. 1. Immunoblot analysis of envelope proteins of different avian hepadnavirus strains with monoclonal antibody SD20. (A) Partially purified viral particles of DHBV Fm, Fp, C, and Am (lanes 1 to 4, respectively); (B) protein extracts of liver tissue infected with HHBV and with DHBV Fm, Fp, C, 26, 3, and 1 (lanes 1 to 7, respectively). For comparison, viral particles of serum containing DHBV Fp were loaded in lane 8.

SD20 recognizes a pre-S epitope conserved in DHBV isolates. To test whether SD20 recognizes an epitope conserved among avian hepadnaviruses, we used immunoblotting to analyze the reactivity of the pre-S proteins in sera and livers of different DHBV isolates and from an HHBV-infected heron.

One major protein (36 kDa) and, with variable intensity, a protein of 28 kDa reacted when we used concentrated DHBV virion preparations from sera of ducks infected with DHBV Fm, DHBV Fp, DHBV C, and DHBV Am (Fig. 1A). In addition, several minor bands ranging from 28 to 38 kDa were observed (Fig. 1A). In the liver samples, the pre-S protein pattern was slightly different, as both the 36- and 28-kDa bands were present as major immunoreactive bands (Fig. 1B). In contrast, pre-S proteins of HHBV-infected livers were not detected by SD20 (Fig. 1B). Infection of the heron liver was independently demonstrated by the presence of large amounts of nucleocapsid protein as determined by immunoblotting with anti-core antibodies and by detection of viral DNA by Southern blot analysis (data not shown). This suggests that SD20 recognizes a pre-S epitope that is conserved in DHBVs but absent in HHBV pre-S protein.

Localization of the pre-S epitope. To map the binding site of SD20, we tested its reactivity by immunoblotting to several MS2-pre-S fusion proteins expressed in *E. coli* (Fig. 2). Some of the fusion proteins were efficiently expressed in *E.*

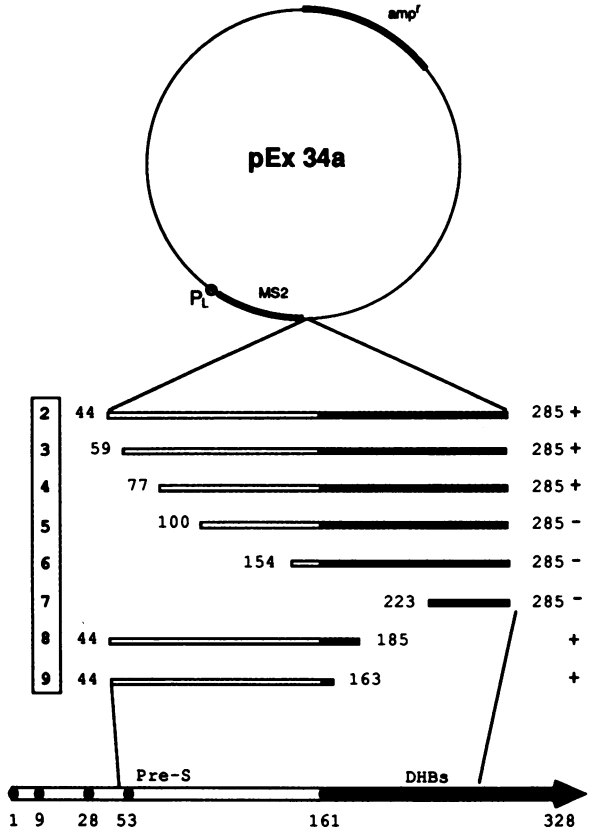

FIG. 2. DHBV pre-S/S proteins expressed in *E. coli*. Fragments of cloned DHBV 16 DNA (60) inserted into expression vector pEx 34a with the amino acid positions of the pre-S/S gene. Numbered black dots in the pre-S/S gene indicate positions of the AUG codons. The reaction pattern of the corresponding fusion proteins with the SD20 monoclonal antibody is indicated in the right panel. The boxed numbers denoting fusion proteins (left-hand side) correspond to the lanes of the immunoblot of Fig. 3 into which the cell extracts were loaded.

coli and could be visualized by Coomassie blue staining (data not shown); others were detected only by immunoblotting with an anti-MS2 monoclonal antibody (Fig. 3A). The deduced molecular weights of the fusion proteins with the lowest electrophoretic mobility were in close agreement with those predicted from the sequence. Proteins with higher electrophoretic mobility which were also detected probably derive from degradation during synthesis in *E. coli* or premature termination of translation or from partially degraded mRNA. SD20 reacted with five of the eight fusion proteins tested (Fig. 3B). The reaction pattern observed indicates that the epitope is localized between amino acids 77 and 100 of the pre-S polypeptide (Fig. 2). This region is in a highly conserved pre-S domain of all DHBV isolates, containing only one point mutation in one isolate (Fig. 4).

In vitro neutralizing activity. To examine whether SD20 would block infectivity of DHBV, we performed an in vitro neutralization test. Primary duck hepatocytes were infected with DHBV-positive duck serum (MOI of 280 and 28) with or without previous incubation with SD20 ascites fluid. The ascites fluid was used in excess (1:200 dilution) as determined by the method of limiting dilution (data not shown). Intracellular viral DNA was examined 9 days after infection, and DNA from secreted virions was determined every 2 days.

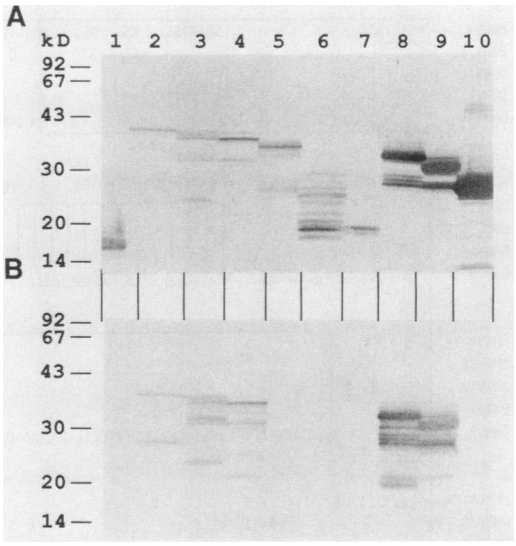

FIG. 3. Mapping of the epitope recognized by monoclonal antibody SD20. MS2 fusion proteins expressed in *E. coli*, separated by SDS-polyacrylamide gel electrophoresis, and blotted to nitrocellulose were visualized by an MS2-specific monoclonal antibody (A) and tested for reactivity with monoclonal antibody SD20 (B). As negative controls, the MS2 protein expressed by the vector alone and an MS2-DHBV polymerase fusion protein were loaded in lanes 1 and 10, respectively. In lanes 2 to 9 MS2-pre-S/S fusion proteins as indicated in Fig. 2 were loaded.

At an MOI of 28, several bands of intracellular viral DNA characteristic of viral replication were detected (Fig. 5A, lane c), indicating that active DHBV replication occurs in these cells. When the virus was preincubated with SD20, the amount of intracellular viral DNA was strongly reduced (73.4 ± 5.1%) (Fig. 5A, lanes c and d) as quantified by dot spot hybridization and radioactivity counting (data not shown). Neutralization was as effective (73.6 ± 1.4%) when a 10-fold-higher MOI was tested (Fig. 5A, lanes a and b). In the medium, the release of DHBV particles was detectable from day 5 until day 9 for both MOIs tested. Similarly, as observed inside the cells, DHBV DNA released into the medium was decreased by approximately 75% (Fig. 5B). Incubation of the inoculum with an unrelated ascites fluid did not affect the DHBV DNA titer in cells or supernatants (data not shown), which demonstrates the specificity of the in vitro neutralization assay. The neutralization capacity of the other anti-DHBV monoclonal antibodies is currently being investigated.

DISCUSSION

In this paper a monoclonal antibody, SD20, with neutralizing activity against pre-S protein of DHBV has been described. Although the neutralizing activity of SD20 was high (75%), complete neutralization was never achieved. A constant residual infectivity of 25% was observed under several assay conditions and was not changed by 10-fold dilution of the inoculum. Interference of very abundant duck serum proteins in the binding of the SD20 antibody to viral particles can therefore not explain the residual infectivity. The concentration of the SD20 was not a limiting factor, since the neutralization assay had been performed in an excess of antibody. Our observation of only partial neutralization is consistent with related studies which showed that

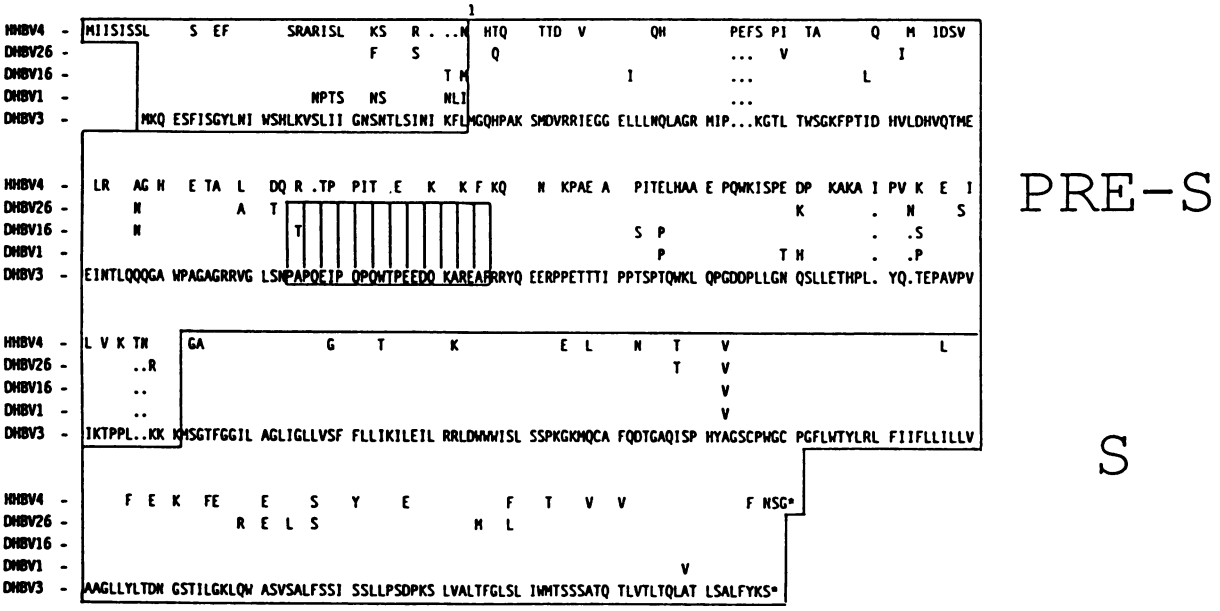

FIG. 4. Pre-S/S protein sequence alignment of several avian hepadnaviruses. The pre-S/S proteins were translated from the nucleotide sequence of HHBV and of various cloned DHBVs (see Materials and Methods for sources). The DHBV isolate of German origin (DHBV 3 [60]) is given on the bottom line, and amino acid differences from the other avian hepadnaviruses are shown in the corresponding upper rows. To maximize the number of matches, a few amino acid insertions had to be made (indicated by dots). The AUG believed to be used as translation initiation codon for the major 36-kDa pre-S protein (2) is numbered as 1. The antigenic region to which the monoclonal antibody SD20 binds is indicated by the striped box.

for virtually all viruses a small fraction escapes neutralization even when a large excess of neutralizing antibodies is used (37). Several factors such as neutralization escape variants, aggregation of viral particles, and retention of virus infectivity despite the presence of bound antibody, have been proposed to explain the persistent fraction of infecting virus (9, 26, 37). Furthermore, since the neutralization test requires 9 days, there is time for horizontal spread of virus, which may prevent the measurement of more effective neutralization.

During preparation of this article, another report describing DHBV pre-S monoclonal antibodies with neutralizing activity came to our attention (3). In this study, neutralizing monoclonal antibodies recognizing three nonoverlapping epitopes were identified on the DHBV pre-S protein, but no precise localisation was attempted. Like SD20, they reacted with three pre-S proteins of 34, 36, and 37 kDa, but unlike SD20, the 28-kDa pre-S protein was not detected. This suggests that SD20 does not bind to the same epitope, although it may bind within the same region.

Two major conclusions can be drawn from both independently performed neutralization studies. First, DHBV pre-S proteins are targets for neutralizing antibodies; second, some pre-S sequences are located at accessible sites on the surface of viral particles. Similar conclusions have been drawn for pre-S proteins of HBV (1, 12, 24, 42, 43, 46) and are also supported by the finding that protective immunity can be successfully induced by immunization of chimpanzees with synthetic peptides and that pre-S-specific antibodies develop during self-limited hepatitis in humans (15, 27, 28). However, neither of these observations proves the neutralizing activity of HBV pre-S specific antibodies alone, because protective immunity might have been induced or complemented by cellular immune defense mechanisms and pre-S antibody induction during virus elimination may be coincidental. In contrast, the in vitro neutralization studies performed with DHBV-infected primary duck hepatocytes provide unequivocal evidence for the neutralization activity of the corresponding antibodies alone.

Substantial information is now available on the surface location of HBV pre-S sequences and their accessibility to antibodies (1, 42, 43, 46). A sequence close to the amino terminus of pre-S1 which is implicated in host cell receptor binding (amino acids 20 to 50), a major immunogenic region upstream of the pre-S2 initiation codon (amino acids 94 to 117), and most of the pre-S2 sequences are probably located on the surface of the viral particles. However, the primary structure of DHBV pre-S sequence shows too little sequence homology with the pre-S sequence of HBV (36, 60) to permit a comparison with any of the exposed surface sites of HBV. From the studies with HBV, it appears likely that most of the pre-S sequence is exposed at the surface of the viral particles, and this could be similar for DHBV pre-S protein.

The pre-S region contains a total of five or six in-frame AUGs (depending on the subtype [Fig. 4]). Only one major putative DHBV pre-S mRNA has been described, from which synthesis of a pre-S protein of 36 kDa initiated at the second AUG of the pre-S frame is predicted (2). Interestingly, however, immunoblotting with SD20 revealed several pre-S proteins ranging from 28 to 37 kDa. Three DHBV pre-S proteins of 34, 35 or 36, and 37 kDa were also observed in four independent studies with polyclonal and monoclonal antibodies (3, 34, 54, 68). The 36- and 37-kDa pre-S proteins could represent the myristylated and nonmyristilated forms of the same molecule as described for HBV (48). Their synthesis was recently shown by transfection of mutant DHBV genomes into HepG2 cells to initiate at the second AUG (at viral nucleotide 801) of the pre-S region (D. Fernholz and H. Will, unpublished data). Unlike the antibodies used by Schlicht et al. (54) and Cheung et al. (3),

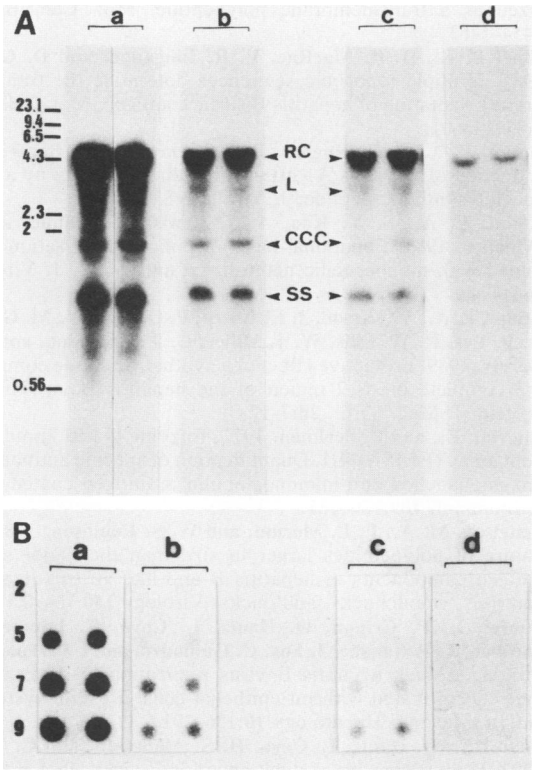

FIG. 5. Neutralization of DHBV with monoclonal antibody SD20 in primary hepatocyte cell culture. Viral DNA in the cells at the end of the culture was monitored by Southern blotting (A), and viral DNA in the cell culture medium was monitored every 2 days postinfection (as indicated) by spot hybridization (B). One day after plating, the hepatocytes were infected with DHBV-positive serum at an MOI of 280 (lanes a and b) and 28 (lanes c and d) with (lanes b and d) or without (lanes a and c) prior incubation with SD20 ascites fluid. On the Southern blot the positions of the size markers (*Hin*dIII-digested lambda DNA) and the various forms of DHBV replicative intermediates are indicated (RC, relaxed circular; L, linear; CCC, covalently closed circular; and SS, single stranded). No hybridization signals were obtained with DNA from uninfected cells (data not shown).

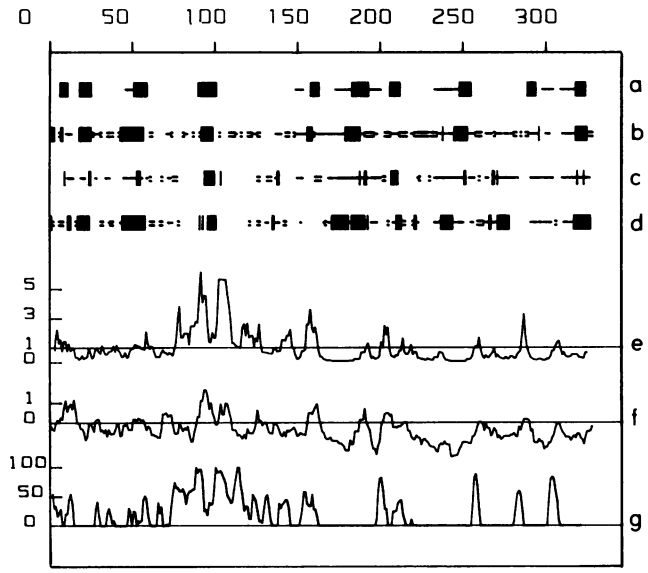

FIG. 6. Computer-assisted prediction of antigenic sites. The DHBV pre-S protein sequence as predicted from cloned viral DHBV 16 DNA starting at nucleotide 801 and ending at nucleotide 1785 (nomenclature as in reference 36) was used. The secondary structures were predicted according to Chou and Fasman (4) (line a), Garnier et al. (20) (line b), Deléage and Roux (8) (line c), and Levin et al. (33) (line d). The given conformational states are α-helix (▬), β-sheet (—) and turn (=). The blank regions indicate aperiodic states. Lines e, f, and g are profiles generated with different computer programs (13, 25, 47) predicting potential antigenic sites of proteins. Peaks above the *x* axis indicate the most solvent accessible (line e), hydrophilic (line f), and antigenic (line g) regions. For each graph the scale is given on the *y* axis.

SD20 also reacted with several pre-S proteins smaller than 34 kDa (Fig. 1). The most prominent of these proteins was a 28-kDa protein. Consistent with our observation, a 28-kDa protein was also seen by immunoblotting with two polyclonal sera produced by immunization of rabbits with pre-S proteins derived from viral particles (68). Although these data strongly suggested that p28 is a pre-S protein, the immunoreactivity of p28 with an antibody to another virus-encoded protein which might have been induced by a contaminating antigen used for immunization was not rigorously excluded. The data obtained with SD20 and, especially, the immunoreactivity of the 28-kDa protein with a polyclonal antibody to a recombinant pre-S protein expressed in *E. coli* (Fernholz and Will, unpublished), as well as with the polyclonal antibodies to purified viral particles (68), establish unequivocally the 28-kDa molecule as a new viral protein which contains DHBV pre-S sequences. The binding site of SD20 on the pre-S polypeptide as mapped with the recombinant pre-S proteins expressed in *E. coli* indicates that pre-S sequences between amino acids 77 and 100 are contained in the 28-kDa protein. p28 could be initiated at an internal AUG (as speculated previously [68]) or at an unconventional codon of the pre-S region; it could represent a proteolytic processing product, or it could derive from a spliced mRNA which has not been detected so far. A protein of 30 kDa reacting with SD20 is present in variable amounts in some infected livers and virus pellets and may be initiated at the AUG codon at nucleotide 957, which would predict a 30-kDa pre-S protein. The more complex pre-S protein pattern seen in serum than that in infected livers is intriguing. More efficient proteolytic degradation of pre-S proteins in serum than in liver could be a trivial explanation.

A hydrophilicity profile indicates that the DHBV pre-S region is rather hydrophilic, whereas the S region contains two major hydrophobic peaks (Fig. 6). As speculated for mammalian hepadnaviruses, the hydrophobic domains of the S protein may be involved in its anchoring to the cell membrane, which is reminiscent of transmembrane proteins of other viruses (11). The main antigenic site of DHBV as predicted by computer algorithms is located in the pre-S region between amino acids 75 and 110 (Fig. 6). Consistent with the computer prediction, the epitope recognized by SD20 is localized between amino acids 77 and 100 (Fig. 5). The sequence divergence of the DHBV and HHBV pre-S proteins is rather high (51%) and has been speculated to be at least partly responsible for the different host ranges of the two viruses (58). However, the predicted hydrophilicity profile and the antigenic profile were almost indistinguishable (data not shown), suggesting that conservation of certain hydrophilic domains is of functional importance. SD20 binds to a region which exhibits a highly conserved 6- to 8-amino-acid helix (amino acids 92 to 100) as predicted by all

algorithms used. A similar but longer helix (amino acids 86 to 103) is also present in the corresponding HHBV pre-S region (data not shown).

The availability of neutralizing monoclonal antibodies provides a useful tool for investigating mechanisms of virus neutralization. Despite extensive studies with synthetic peptides and anti-peptide antibodies which allowed the mapping of putative virus recognition sites for cell receptors within the pre-S region of HBV, elucidation of the mechanisms of virus neutralization remained elusive. It appears from these studies that the S protein has no receptor recognition site and the pre-S2 region has only an auxiliary role in HBV binding to hepatocytes (43, 46). However, it is known that S and pre-S2 proteins elicit protective antibodies in humans and chimpanzees (15, 21, 27, 46, 51). Thus, neutralization of HBV appears complex and can also be brought about by mechanisms other than direct interference with the receptor-binding site, as is known for other viruses (9, 37). For hepadnaviruses, DHBV is the only convenient model which can be used to decipher the mechanisms of virus neutralization both in vivo and in vitro.

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LITERATURE CITED

1. **Budkowska, A., M. M. Riottot, P. Dubreuil, Y. Lazizi, C. Brechot, E. Sobczak, M. A. Petit, and J. Pillot.** 1986. Monoclonal antibody recognizing pre-S 2 epitope of hepatitis B virus: characterization of pre-S 2 epitope and anti-pre-S 2 antibody. J. Med. Virol. **20**:111–125.
2. **Büscher, M., W. Reiser, H. Will, and H. Schaller.** 1985. Transcripts and the putative RNA pregenome of duck hepatitis B virus: implications for reverse transcription. Cell **40**:717–724.
3. **Cheung, R. C., W. S. Robinson, P. L. Marion, and H. B. Greenberg.** 1989. Epitope mapping of neutralizing monoclonal antibodies against duck hepatitis B virus. J. Virol. **63**:2445–2451.
4. **Chou, G. Y., and G. D. Fasman.** 1978. Prediction of secondary structure of proteins from amino acid sequence. Adv. Enzymol. Relat. Areas Mol. Biol. **47**:145–148.
5. **Cova, L., O. Hantz, M. Arliaud-Gassin, A. Chevalier, P. Berthillon, L. Boulay, C. Jacquet, B. Chomel, L. Vitvitski, and C. Trépo.** 1985. Comparative study of DHBV DNA levels and endogenous DNA polymerase activity in naturally infected ducklings in France. J. Virol. Methods **10**:251–260.
6. **Cova, L., V. Lambert, A. Chevalier, O. Hantz, I. Fourel, C. Jacquet, C. Pichoud, J. Boulay, B. Chomel, L. Vitvitski, and C. Trépo.** 1986. Evidence for the presence of duck hepatitis B virus in wild migrating ducks. J. Gen. Virol. **67**:537–547.
7. **Deléage, G., F. F. Clerc, B. Roux, and D. C. Gautheron.** 1988. Antheprot: a package for protein sequence analysis using a microcomputer. Comput. Appl. Biosci. **4**:351–356.
8. **Deléage, G., and B. Roux.** 1987. A algorithm for protein secondary structure prediction based on class prediction. Protein Eng. **1**:289–294.
9. **Dimmock, N. J.** 1984. Mechanism of virus neutralization. J. Gen. Virol. **65**:1015–1022.
10. **Dreesman, G. R., Y. Sanchez, I. Ionescu-Matiu, J. T. Sparrow, H. R. Six, D. L. Peterson, F. B. Hollinger, and J. L. Melnick.** 1982. Antibody to hepatitis B surface antigen after a single inoculation of uncoupled synthetic HBsAg peptides. Nature (London) **295**:158–160.
11. **Eble, B. E., V. R. Lingappa, and D. Ganem.** 1986. Hepatitis B surface antigen: an unusual secreted protein initially synthesized as a transmembrane polypeptide. Mol. Cell. Biol. **6**:1454–1463.
12. **Eble, B. E., D. R. MacRae, V. R. Lingappa, and D. Ganem.** 1987. Multiple topogenic sequences determine the transmembrane orientation of hepatitis B surface antigen. Mol. Cell. Biol. **7**:3591–3601.
13. **Emini, E. A., J. V. Hughes, D. S. Perlow, and J. Boger.** 1985. Induction of hepatitis A virus-neutralizing antibody by a virus-specific synthetic peptide. J. Virol. **55**:836–839.
14. **Emini, E. A., S. Y. Kao, A. J. Lewis, R. Crainic, and E. Wimmer.** 1983. Functional basis of polio neutralization determined with monospecific neutralizing antibodies. J. Virol. **46**:466–474.
15. **Emini, E. A., V. Larson, J. Eichberg, P. Conard, V. M. Garsky, D. R. Lee, R. W. Ellis, W. J. Miller, C. A. Anderson, and R. J. Gerety.** 1989. Protective effect of a synthetic peptide comprising the complete pre-S 2 region of the hepatitis B virus surface protein. J. Med. Virol. **28**:7–12.
16. **Engvall, E., and P. Perlman.** 1972. Enzyme-linked immunosorbent assay (ELISA). III. Quantification of specific antibodies by enzyme-labelled anti-immunoglobulin in antigen coated tubes. J. Immunol. **109**:129–135.
17. **Feitelson, M. A., P. L. Marion, and W. S. Robinson.** 1983. The nature of polypeptides larger in size than the major surface antigen components of hepatitis B and like viruses in ground squirrels, woodchucks, and ducks. Virology **130**:76–90.
18. **Fourel, I., P. Gripon, O. Hantz, L. Cova, V. Lambert, C. Jacquet, K. Watanabe, J. Fox, C. Guillouzo, and C. Trépo.** 1989. Prolonged duck hepatitis B virus replication in duck hepatocytes cocultivated with rat epithelial cells: a useful system for antiviral testing. Hepatology **10**:186–191.
19. **Fourel, I., O. Hantz, L. Cova, H. S. Allaudeen, and C. Trépo.** 1987. Main properties of duck hepatitis B virus DNA polymerase: comparison with the human and woodchuck hepatitis B virus DNA polymerases. Antiviral Res. **8**:189–199.
20. **Garnier, J., D. J. Osguthorpe, and B. Robson.** 1978. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. J. Mol. Biol. **120**:97–120.
21. **Gerin, J. L., H. Alexander, J. W.-K. Shih, R. H. Purcell, G. Dapolito, R. Engle, N. Green, J. G. Sutcliffe, T. M. Shinnick, and R. A. Lerner.** 1983. Chemically synthesized peptides of hepatitis B surface antigen duplicate the d/y specificities and induce subtype-specific antibodies in chimpanzees. Proc. Natl. Acad. Sci. USA **80**:2365–2369.
22. **Guldner, H. H., H. J. Netter, C. Szostecki, H. J. Lakomek, and H. Will.** 1988. Epitope mapping with a recombinant human 68 kDa (U1) ribonucleoprotein antigen reveals heterogeneous autoantibody profiles in human autoimmune sera. J. Immunol. **141**:469–475.
23. **Gust, I. D., A. G. Coulepsis, W. S. Robinson, and A. J. Zuckerman.** 1986. Taxonomic classification of human hepatitis B virus. Intervirology **25**:14–29.
24. **Heermann, K.-H., F. Kruse, M. Seifer, and W. H. Gerlich.** 1987. Immunogenicity of the gene S and pre-S domains in hepatitis B virions and HBsAg filaments. Intervirology **28**:14–25.
25. **Hopp, T. P., and K. R. Woods.** 1981. Prediction of protein antigenic determinants from amino acid sequences. Proc. Natl. Acad. Sci. USA **78**:3824–3828.
26. **Iorio, R. M., and M. A. Bratt.** 1985. Neutralization of Newcastle disease virus by monoclonal antibodies to the hemagglutinin-neuraminidase glycoprotein: requirement for antibodies to four sites for complete neutralization. J. Virol. **51**:445–451.
27. **Itoh, Y., E. Takai, H. Ohnuma, K. Kitayima, F. Tsuda, A. Machida, S. Mishiro, T. Nakamura, Y. Miyakawa, and M. Mayumi.** 1986. A synthetic peptide vaccine involving the product of the pre-S 2 region of hepatitis B virus: protective efficacy in chimpanzees. Proc. Natl. Acad. Sci. USA **83**:9174–9178.
28. **Klinkert, M. Q., L. Theilmann, E. Pfaff, and H. Schaller.** 1986. Pre-S 1 antigens and antibodies early in the course of acute hepatitis B virus infection. J. Virol. **58**:522–525.
29. **Köhler, G., and C. Milstein.** 1976. Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion.

Eur. J. Immunol. 6:511–519.
30. **Kyhse-Andersen, J.** 1984. Electroblotting of multiple gels using a simple apparatus without buffer tank for rapid transfer of protein from polyacrylamide to nitrocellulose. J. Biochem. Biophys. Methods 10:203–209.
31. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680–685.
32. **Lambert, V., L. Cova, W. R. Hansen, B. Chomel, and C. Trépo.** 1988. Duck hepatitis B virus in wild waterfowl from the United States, p. 523–525. *In* A. J. Zuckerman (ed.), Viral hepatitis and liver disease. Alan R. Liss, Inc., New York.
33. **Levin, J. M., B. Robson, and J. Garnier.** 1986. An algorithm for secondary structure determination in proteins based on sequence similarity. FEBS Lett. 205:303–308.
34. **Li, J. S., L. Cova, R. Buckland, V. Lambert, G. Deléage, and C. Trepo.** 1989. Duck hepatitis B virus can tolerate insertion, deletion and partial frameshift mutation in the distal pre-S region. J. Virol. 63:4965–4968.
35. **Machida, A., S. Kishimoto, H. Ohnuma, K. Baba, Y. Ito, Miyamoto, G. Funatsu, K. Oda, S. Usuda, S. Togami, T. Nakamura, Y. Miyakawa, and M. Mayumi.** 1984. A polypeptide containing 55 amino acid residues coded by the pre-S region of hepatitis B virus deoxyribonucleic acid bears the receptor for polymerized human as well as chimpanzee albumin. Gastroenterology 86:910–918.
36. **Mandart, E., A. Kay, and F. Galibert.** 1984. Nucleotide sequence of a cloned duck hepatitis B virus genome: comparison with woodchuck and human hepatitis B virus sequences. J. Virol. 49:782–792.
37. **Mandel, B.** 1984. Mechanism of virus neutralization, p. 32–38. *In* A. L. Notking and M. B. A. Oldstone (ed.), Concepts in viral pathogenesis. Springer-Verlag, New York.
38. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
39. **Marion, P. L., S. S. Knight, M. A. Feitelson, L. S. Oshiro, and W. S. Robinson.** 1983. Major polypeptide of duck hepatitis B surface antigen particles. J. Virol. 48:534–541.
40. **Marion, P. L., L. S. Oshiro, D. C. Regenery, G. H. Scullard, and W. S. Robinson.** 1980. A virus in Beechey ground squirrels that is related to hepatitis B virus of man. Proc. Natl. Acad. Sci. USA 77:2941–2945.
41. **Mason, W. S., G. Seal, and J. Summers.** 1980. A virus of Pekin ducks with structural and biological relatedness to human hepatitis B virus. J. Virol. 36:829–836.
42. **Milich, D. R.** 1987. Genetic and molecular basis for T- and B-cell recognition of hepatitis B viral antigens. Immunol. Rev. 99: 71–103.
43. **Neurath, A. R., and S. B. H. Kent.** 1989. The pre-S region of hepadnavirus envelope proteins. Adv. Viral oncol. 34:64–142.
44. **Neurath, A. R., S. B. H. Kent, K. Parker, A. M. Prince, N. Strick, B. Brotman, and P. Sproul.** 1986. Antibodies to a synthetic peptide from the pre-S 120–145 region of the hepatitis B virus envelope are virus neutralizing. Vaccine 4:35–37.
45. **Neurath, A. R., S. B. H. Kent, N. Strick, and K. Parker.** 1986. Identification and chemical synthesis of a host cell receptor binding site on hepatitis B virus. Cell 46:429–436.
46. **Neurath, A. R., N. Strick, S. B. H. Kent, K. Parker, C. S. Kim, M. Girard, H. E. Ralph, and J. Valinsky.** 1989. Biological role of pre-S sequences of the hepatitis B virus (HBV) envelope protein, p. 143–158. *In* J. P. Tam and E. T. Kaiser (ed.), Synthetic peptides: approaches to biological problems. Alan R. Liss, Inc., New York.
47. **Parker, J. M. R., D. Guo, and R. S. Hodges.** 1986. New hydrophilicity scale derived from high performance liquid chromatography peptide retention data: correlation of predicted surface residues with antigenicity and X-ray-derived accessible sites. Biochemistry 25:5425–5432.
48. **Persing, D. H., H. E. Varmus, and D. Ganem.** 1987. The pre-S 1 protein of hepatitis B is acylated at its amino terminus with myristic acid. J. Virol. 61:1672–1677.
49. **Pontisso, P., M.-A. Petit, M. J. Bankowski, and M. E. Peeples.** 1989. Human liver plasma membranes contain receptors for the hepatitis B virus pre-S1 region and, via polymerized human serum albumin, for the pre-S2 region. J. Virol. 63:1981–1988.
50. **Pugh, J. C., J. J. Sninsky, J. W. Summers, and E. Schaeffer.** 1987. Characterization of a pre-S polypeptide on the surfaces of infectious avian hepadnavirus particles. J. Virol. 61:1384–1390.
51. **Purcell, R. H., and J. L. Gerin.** 1985. Prospects for a second and third generation of hepatitis B vaccines. Hepatology 5:159–163.
52. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. 113:237–251.
53. **Schaeffer, E., and J. J. Sninsky.** 1984. Predicted secondary structure similarity in the absence of primary amino acid sequence: hepatitis B virus open reading frames. Proc. Natl. Acad. Sci. USA 81:2902–2906.
54. **Schlicht, H. J., C. Kuhn, B. Guhr, R. J. Mattaliano, and H. Schaller.** 1987. Biochemical and immunological characterization of the duck hepatitis B virus envelope proteins. J. Virol. 61:2280–2285.
55. **Schödel, F., R. Sprengel, T. Weimer, D. Fernholz, R. Schneider, and H. Will.** 1989. Animal hepatitis B viruses. Adv. Viral Oncol. 8:73–102.
56. **Schulman, M., C. D. Wilde, and G. Köhler.** 1978. A better cell line for making hybridomas secreting specific antibodies. Nature (London) 276:269–270.
57. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503–517.
58. **Sprengel, R., E. F. Kaleta, and H. Will.** 1988. Isolation and characterization of a hepatitis B virus endemic in herons. J. Virol. 62:3832–3839.
59. **Sprengel, R., C. Kuhn, C. Manso, and H. Will.** 1984. Cloned duck hepatitis B virus DNA is infectious in Pekin ducks. J. Virol. 52:932–937.
60. **Sprengel, R., C. Kuhn, H. Will, and H. Schaller.** 1985. Comparative sequence analysis of duck and human hepatitis B virus genomes. J. Med. Virol. 15:323–333.
61. **Sprengel, R., and H. Will.** 1987. Duck hepatitis B virus, p. 363–386. *In* G. Darai (ed.), Virus disease in laboratory and captive animals. Martinus Nijhoff Publishing, Boston.
62. **Strebel, K., E. Beck, K. Strohmeyer, and H. Schaller.** 1986. Characterization of foot-and-mouth disease virus gene products with antisera against bacterially synthesized fusion proteins. J. Virol. 57:983–990.
63. **Summers, J. W., J. M. Smolec, and R. Snyder.** 1978. A virus similar to hepatitis B virus associated with hepatitis and hepatoma in woodchucks. Proc. Natl. Acad. Sci. USA 75:4533–4537.
64. **Tuttleman, J. S., J. C. Pugh, and J. W. Summers.** 1986. In vitro experimental infection of primary duck hepatocyte cultures with duck hepatitis B virus. J. Virol. 58:17–25.
65. **Vitvitski, L., M. L. Meyers, J. J. Sninsky, P. Berthillon, P. Chevalier, M. A. Sells, G. Acs, and C. Trépo.** 1988. Expression of the X product of hepatitis B virus and WHV in infected livers and transfected 3T3 cells: evidence for cross reactivity and correlation with core/e gene expression, p. 341–344. *In* A. J. Zuckerman (ed.), Viral hepatitis and liver disease. Alan R. Liss, Inc., New York.
66. **Voller, A., D. Bidwell, and A. Barlett.** 1976. Microplate enzyme immunoassay for immunodiagnosis of virus infections, p. 506–512. *In* N. R. Rose and H. Friedman (ed.), Manual of clinical immunology, 1st ed. American Society for Microbiology, Washington, D.C.
67. **Wahl, G. M., M. Stern, and G. R. Stark.** 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxmethyl-paper and rapid hybridization by using dextran sulfate. Proc. Natl. Acad. Sci. USA 76:3683–3687.
68. **Yokosuka, O., M. Omata, and Y. Ito.** 1988. Expression of pre-S1, pre-S2, and C proteins in duck hepatitis B virus infection. Virology 167:82–86.
69. **Zhou, Y. Z.** 1980. A virus possibly associated with hepatitis and hepatoma in ducks. Shanghai Med. J. 3:641–644.