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Immunogenic Domains of Hepatitis Delta Virus Antigen: Peptide Mapping of Epitopes Recognized by Human and Woodchuck Antibodies

JIA-GANG WANG,[1,2] ROBERT W. JANSEN,[1] EDWIN A. BROWN,[1] AND STANLEY M. LEMON[1]*

Division of Infectious Diseases, Department of Medicine, School of Medicine,[1] and Department of Parasitology and Laboratory Practice, School of Public Health,[2] University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7030

Received 8 May 1989/Accepted 7 November 1989

Hepatitis delta virus (HDV) is a defective RNA virus which is dependent on hepatitis B virus for essential helper functions. Only a single highly basic phosphoprotein, HDV antigen (HDAg), is expressed by the HDV genome during infection in humans. Antibody directed to HDAg is important in the diagnosis of HDV infection, and it is likely but not yet proven that the immune response to HDAg provides significant protection against subsequent exposures to HDV. In an effort to map the antigenic domains of HDAg, 209 overlapping hexapeptides, spanning the entire 214 amino acid residues of the protein, were synthesized on polyethylene pins and probed by enzyme-linked immunosorbent assay with sera containing high titers of anti-HD antibodies. Domains recognized by antibodies present in serum from human chronic carriers of this virus included residues 2 to 7, 63 to 74, 86 to 91, 94 to 100, 159 to 172, 174 to 195, and 197 to 207. Antibody from an acutely superinfected woodchuck recognized similar epitopes, as well as a domain spanning residues 121 to 128. Together, residues in these antigenic domains constitute 41% of the HDAg molecule. Oligopeptides 15 to 29 residues in length and representing epitopes of HDAg found to be dominant in humans (residues 2 to 17, 156 to 184, and 197 to 211) were synthesized in bulk and found to possess significant antigenic activity by microdilution enzyme-linked immunosorbent assay. The reactivity of peptide 197–211 with human sera confirms that the entire 214 amino acids of HDAg are expressed during infection in vivo. In addition, these results suggest that synthetic peptides may be useful reagents for development of new and improved diagnostic tests for HDV infection.

Hepatitis delta virus (HDV) is a unique human pathogen which has been implicated, in association with hepatitis B virus (HBV), as a cause of severe acute hepatitis and progressive chronic liver disease (7). The HDV virion consists of a circular, single-stranded RNA genome approximately 1.7 kilobases in length (17, 19, 26, 27) and a highly basic phosphoprotein (hepatitis delta antigen [HDAg]) (4, 5) packaged within an envelope composed of hepatitis B surface antigen (HBsAg) (3, 24). HBsAg present in the HDV virion is encoded by HBV DNA, and current data indicate the HBV coinfection is an absolute prerequisite for significant infection and disease associated with HDV in humans.

Complete genomic sequences have been independently derived from cDNA clones established from two different HDV strains, one after chimpanzee passage (26, 27) and the other directly from human serum (19). Analysis of these sequences has shown that virion RNA contains two open reading frames (ORF) capable of encoding proteins of longer than 100 amino acids, while the antigenomic sequence contains three additional open reading frames of equivalent length (19, 26, 27). Thus, the HDV genome potentially encodes up to five different proteins of significant length. HDAg has been shown to be encoded by one of the open reading frames in antigenomic sense RNA (5, 28): ORF-5 by the nomenclature of Wang et al. (26, 27), or ORF-2 by Makino et al. (19). Thus, HDV is a negative-stranded RNA virus. HDAg is present in HDV particles in two distinct forms having molecular weights of 27,000 ($p27^{\delta}$) and 24,000 ($p24^{\delta}$) (3, 28). Present knowledge of the differences between these two HDAg species is incomplete. ORF-5 potentially encodes a protein 214 amino acids in length, thought to represent $p27^{\delta}$ (19, 26, 27). It has been suggested that the $p24^{\delta}$ form of HDAg may represent expression of a C-terminal truncated protein of 195 amino acids, due to heterogeneity in the HDV genome reflected by the presence of an amber stop codon in some cloned cDNAs (28). Alternatively, the two molecular species may reflect posttranslational processing of the primary ORF-5 expression product. HDAg is phosphorylated at multiple serine residues and has been shown to have RNA-binding activity of uncertain specificity (5). Thus far, it is the only protein product clearly recognized to be expressed by the HDV genome during in vivo infection. Further understanding of the structure and function of this viral protein is central to unraveling the molecular events involved in HDV replication and the pathogenesis of delta hepatitis.

In this report, we describe the mapping of immunogenic domains of the HDAg molecule and the identification of epitopes recognized by human and woodchuck antibodies to HDAg (anti-HD). These studies utilized pin-based oligopeptide synthesis as an approach to the fine-structure mapping of sequential epitopes (11). We compare the results of this method with computer algorithms for prediction of protein secondary structure and immunogenic domains.

MATERIALS AND METHODS

Human sera. Sera were collected from hemophiliac patients who were chronic carriers of HBV (HBsAg positive) and enrolled in a longitudinal study exploring the contribution of HDV infection to chronic liver disease in hemophilia.

* Corresponding author.

Control sera were obtained from two healthy individuals of comparable age and sex, one of whom was an anti-HD-negative, chronic HBsAg carrier. Additional sera were collected from a woodchuck (*Marmota monax*), WC862, which was positive for woodchuck hepatitis virus and experimentally superinfected with human HDV in collaboration with John Cullen of the School of Veterinary Medicine, North Carolina State University. Woodchuck hepatitis virus is a hepadnavirus that is closely related to HBV and capable of supplying helper functions necessary for the replication of HDV (22). The anti-HD activity of these sera was determined by a commercially available competitive enzyme-linked immunosorbent assay (ELISA) (Delta EIA; Abbott Laboratories, North Chicago, Ill.). HBsAg was also detected in sera by a commercial ELISA (Auszyme; Abbott Laboratories).

Anti-HD radioimmunoassay. Additional testing for anti-HD was carried out by a microdilution solid-phase competitive radioimmunoassay modified from that described by Rizzetto et al. (25). HDAg used in this assay was extracted from the liver of an acutely superinfected, woodchuck hepatitis virus-positive woodchuck (WC643) by preparing tissue homogenates in 6 M guanidine hydrochloride (pH 6.0), followed by dialysis against phosphate-buffered saline (PBS), as described by Bergmann and Gerin (1). Flexible polyvinyl chloride microdilution plate wells were coated for 2 h at 35°C with 100 μl of a human anti-HD-positive serum (AI035) diluted 1:4,000 in 50 mM carbonate buffer (pH 9.6), washed with PBS containing 0.5% Tween 20 (PBS-T), and loaded with 40 μl of the HDAg preparation. After 2 h of incubation at 37°C, the plates were washed with PBS-T, and 50 μl of a 1:100 dilution of test serum in PBS was added to each well. Following an overnight incubation at 4°C, wells were washed with PBS-T and incubated for 4 h at 4°C with 50 μl of ^{125}I-labeled immunoglobulin G (IgG; 50,000 cpm), isolated from the serum of an anti-HD-positive hemophiliac patient (ET87) by chromatography through DEAE-Sephacryl (Pharmacia, Piscataway, N.J.), iodinated by the chloramine-T method, and diluted in 10% fetal calf serum.

Immunoblot detection of anti-HD. Immunoblots were carried out with HDAg prepared from woodchuck serum, as this source provides antigen of less complexity than that present in liver (1). Serum (1.0 ml) taken from an acutely superinfected woodchuck (WC643) was layered over an 11-ml cushion containing 20% sucrose in 0.02 M HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.4)–0.01 M $CaCl_2$–0.01 M $MgCl_2$–0.1% bovine serum albumin and centrifuged for 5 h at 150,000 × *g* in an SW40 rotor (Beckman Instruments, Inc., Palo Alto, Calif.). Pelleted HDAg was suspended in distilled water and stored at −70°C until use. Antigen diluted in sample buffer (0.0625 M Tris [pH 6.8], 1% sodium dodecyl sulfate, 1% 2-mercapto-ethanol, 10% glycerol, 0.002% bromophenol blue) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with stacking and separating gels containing 4 and 12.5% polyacrylamide, respectively. Separated polypeptides were electrophoretically transferred to nitrocellulose paper at 85 mA for 3 h at 4°C. Nitrocellulose papers were blocked with blocking buffer (3% milk, 50 mM Tris, 150 mM sodium chloride, 5 mM EDTA, 0.25% gelatin, 0.05% sodium azide [pH 7.4]) for 30 min and incubated with test serum diluted 1:1,000 in blocking buffer for 1 h at room temperature. After washing in PBS-T, nitrocellulose papers were incubated for 30 min with horseradish peroxidase-conjugated goat anti-human IgG (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) diluted in PBS. Following an additional washing step, the papers were placed in freshly prepared substrate solution (25 mg of 3,3-diaminobenzidine in 50 ml of 0.05 M Tris, 0.02% hydrogen peroxide [pH 7.4]) for color development.

Pin-based oligopeptide synthesis. Overlapping hexapeptides spanning the entire HDAg molecule were synthesized on polyethylene pins with materials provided as components of the Epitope Mapping Kit manufactured by Cambridge Research Biochemicals, Inc., Valley Stream, N.Y. This method of peptide synthesis uses preformed active ester coupling with 9-fluoroenylmethyl-oxycarbonyl and *t*-butyl-oxycarbonyl protection. Its application to epitope mapping has been extensively described by Geysen et al. (11–13).

Pin-based oligopeptide ELISA. Oligopeptide-bearing pins, disrupted by sonication as described below, were blocked by incubation for 1 h at room temperature in microdilution plates containing 200 μl of blocking buffer per well. Pins were then transferred to a microdilution plate containing, per well, 175 μl of test serum diluted 1:6,000 or more in blocking buffer. Following incubation at 4°C for 24 to 48 h, pins were subjected to three cycles of washing in PBS-T, 30 min per cycle with agitation, and transferred to wells containing horseradish peroxidase-conjugated goat anti-human IgG diluted in blocking buffer without sodium azide. Woodchuck antibody was detected by sequential incubations with rabbit antiserum raised to woodchuck immunoglobulin and horseradish peroxidase-conjugated goat anti-rabbit IgG. After being washed as above, pins were placed in microdilution wells containing 150 μl of freshly prepared substrate solution {25 mg of 2,2′-azino-di-[3-ethylbenzthiazoline sulfonate (6)] (Boehringer-Mannheim Biochemicals, Indianapolis, Ind.) in 50 ml of 0.1 M disodium hydrogen phosphate, 0.08 M citric acid, 0.015% hydrogen peroxide, pH 4.0} and held at room temperature in the dark for 30 min. Color development was stopped by removing the pins, and the A_{405} of the substrate solutions was determined immediately by reading in an automated ELISA plate reader. Data were downloaded to an IBM-XT computer and analyzed, using Lotus 1-2-3 software. Reactions with human sera were considered positive when absorbance was ≥0.2, while woodchuck serum reactions were considered positive when absorbance was ≥0.5. Pins were stripped of immunoglobulin by sonication for 30 min in 1% sodium dodecyl sulfate–0.1% 2-mercaptoethanol–0.1 M sodium dihydrogen orthophosphate at 60°C, washed with hot (60°C) distilled water followed by boiling methanol for 2 min, and air dried.

Bulk synthesis of oligopeptides. Peptide synthesis was carried out with a model 9500 peptide synthesizer (Biosearch, Novato, Calif.), in the Synthetic Peptide Facility of the Department of Microbiology and Immunology, University of North Carolina-Chapel Hill, under the direction of David Klapper. *t*-Butyloxycarbonyl chemistry was used for peptide synthesis (21).

Oligopeptide ELISA for anti-HD. Oligopeptides (750 ng in 75 μl of carbonate buffer) were applied to the wells of flat-bottomed ELISA plates (Falcon; Becton Dickinson Labware, Oknard, Calif.) by incubation for 2 h at 35°C in a humidified chamber. Plates were washed with PBS-T and blocked by the addition of 75 μl of blocking buffer. Serum specimens diluted in blocking buffer (50 μl per well) were added to the plates, which were then incubated overnight at 4°C. Following washing with PBS-T, 50 μl of horseradish peroxidase-conjugated goat anti-human IgG diluted in blocking buffer without sodium azide was added for 50 min at 37°C. Plates were washed with PBS, followed by the addi-

tion of *o*-phenylenediamine substrate solution (55 μl per well) at room temperature for 15 min in the dark. Color development was stopped by the addition of 160 μl; of 1 N H_2SO_4, and the A_{490} was determined by reading in an automated ELISA plate reader.

HDAg secondary-structure predictions. Hydrophilic domains of HDAg were predicted by the method of Hopp and Woods (14), while hydrophobic regions were independently predicted by a program which determined the mean hydrophobicity score for 11 residue windows, using hydrophobic indices derived for individual side chains by Fauchère and Pliška (9). More detailed predictions of the secondary structure of HDAg were done by means of the PEPTIDESTRUCTURE and PLOTSTRUCTURE programs of the University of Wisconsin Genetics Computer Group Sequence Analysis Software Package (15). These programs include predictions of hydrophilicity by the method of Kyte and Doolittle (18), peptide secondary structure by Chou and Fasman (6) and Garnier et al. (10), and surface probability by Emini et al. (8). From these predictive measures, an antigenic index is derived by the algorithm developed by Jameson and Wolf (15). The antigenic index is a measure of the probability that a domain is antigenic and is calculated by summing weighted values for surface accessibility, regional backbone flexibility, and certain features of predicted secondary structure. For comparison with hexapeptide ELISA results and subsequent statistical analysis, the following criteria were used: hydrophilicity, ≥1.3; surface probability, ≥5; flexibility, ≥1.04; antigenic index, ≥1.2; and "turn" or "strong turn" in secondary-structure predictions. In addition, regions predicted to be amphipathic alpha-helices (putative T-cell determinants) were identified by a program developed by Margalit et al. (20).

RESULTS

HDAg epitope mapping with overlapping hexapeptides. We synthesized 209 overlapping hexapeptides spanning the 214 amino acids of human HDAg (ORF-2 expression product) predicted from the nucleotide sequence of cloned HDV cDNA reported by Makino et al. (19). Hexapeptides were synthesized on polyethylene pins, using a synthesis system supplied by Cambridge Research Biochemicals. Control peptides (4-mers) included in the synthesis reacted specifically with murine monoclonal antibody supplied by the manufacturer in subsequent pin-based ELISA (mean signal-to-noise ratio, 4), confirming the validity of the synthesis. However, preliminary pin-based ELISAs demonstrated substantial nonspecific binding of human IgG to pins supporting HDAg hexapeptides at test serum dilutions below 1:2,000. To overcome this problem, we determined the endpoint titer of each of a panel of 27 anti-HD-positive sera in a competitive radioimmunoassay which used as antigen HDAg extracted from woodchuck liver. These sera had been collected from hemophiliac patients who were chronic carriers of HBsAg. We selected for further study five serum samples with particularly high titers of anti-HD, ranging from 1:12,500 to ≥1:312,500 (Table 1). These titers of anti-HD are highly suggestive of chronic infection with HDV. Each of these five serum samples was strongly positive in immunoblot assays against denatured HDAg concentrated from woodchuck serum, reacting with both $p24^{\delta}$ and $p27^{\delta}$ (Fig. 1). We thus considered it likely that these sera had high-titer antibody against sequential epitopes of HDAg that would be detectable in hexapeptide ELISAs. Each serum was tested at a dilution of 1:6,000 to 1:8,000 against the array of HDAg hexapeptides.

TABLE 1. Anti-HD-positive and control human sera

Serum sample	Age of donor (yr)[a]	HBsAg	Anti-HD dilution (titer)[b]	Screening dilution in hexapeptide ELISA
AD062	28	+	1:62,500 (10^{-4})	1:6,000
AF043	24	+	1:12,500 (10^{-3})	1:8,000
AC036	31	+	1:62,500 (10^{-4})	1:8,000
AC039	20	+	≥1:312,500 (10^{-4})	1:8,000
AD099	15	+	1:62,500 (10^{-3})	1:7,000
A008	33	−	<1:100 (<10^{-0})	1:8,000
A073		+	<1:100 (<10^{-0})	1:8,000

[a] All serum samples were from males.

[b] Highest dilution yielding 50% inhibition in competitive inhibition radioimmunoassay with woodchuck liver-derived HDAg (comparative endpoint titer in commercial ELISA).

Results of pin-based hexapeptide ELISAs with one serum specimen (AC036) are shown in Fig. 2. Antibodies present in this serum demonstrated binding activity against hexapeptides representing at least six discrete regions in the linear sequence of the HDAg protein. Epitopes recognized by binding of antibody to hexapeptides spanned residues 2 to 7, 63 to 69, 159 to 165, 167 to 172, 174 to 181, and 201 to 207. Additional hexapeptides demonstrating low-level binding (above background, but less than the arbitrary cut off of absorbance of ≥0.2) represented residues 85 to 91 and 152 to 157 (Fig. 2). These hexapeptides may represent minor determinants. Hexapeptide ELISA results with this positive serum were highly reproducible, while two anti-HD-negative serum samples (A008 and A073, Table 1) yielded consistently negative results (mean absorbance, 0.113 and 0.121; maximum absorbance, 0.176 and 0.157, respectively) when tested against the hexapeptide-bearing pins at similar dilutions (results not shown). Results of assays with alternating positive and negative sera demonstrated that the stripping procedure was effective in completely removing IgG bound

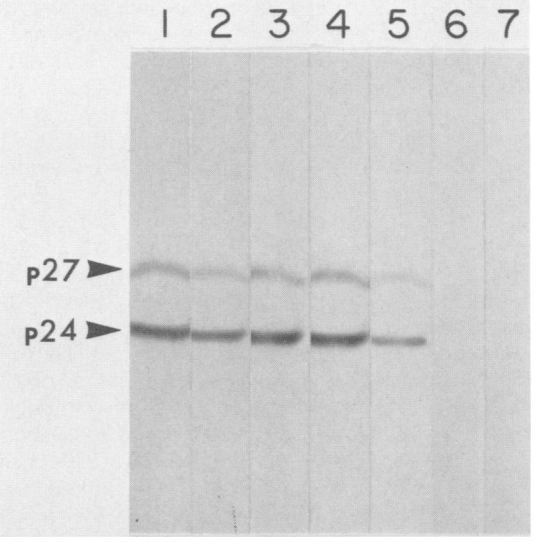

FIG. 1. Immunoblot of woodchuck serum-derived HDAg with human sera utilized in HDAg hexapeptide ELISAs. Lanes 1 to 5 were probed with anti-HD-positive sera (1) AD062, (2) AF043, (3) AC036, (4) AC039, and (5) AD099, while lanes 6 and 7 were probed with control sera A008 and A073, respectively.

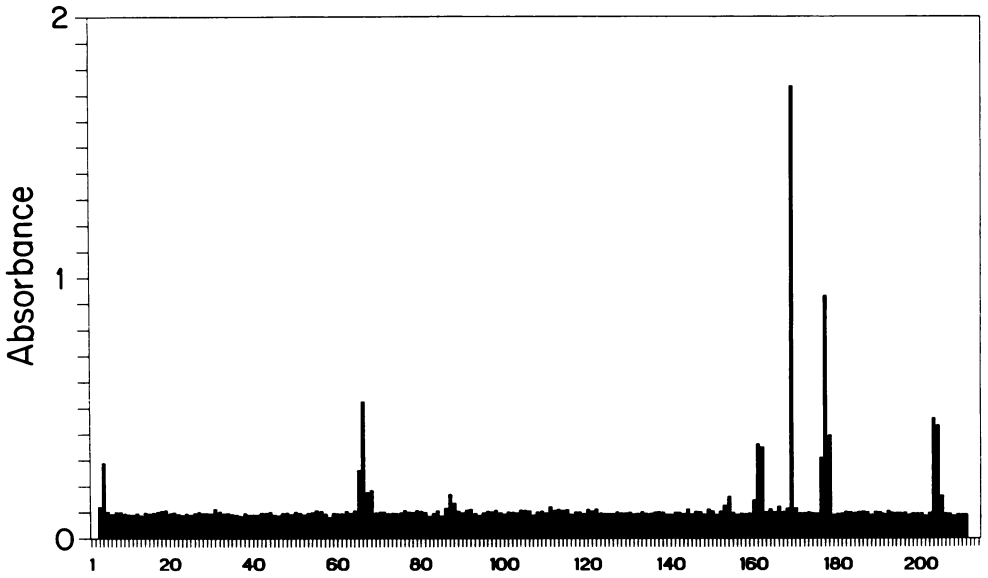

FIG. 2. Hexapeptide ELISA scan of HDAg with serum AC036 at a 1:8,000 dilution. Hexapeptides on pins were considered reactive with antibody when absorbance was ≥0.200.

to the pins. In the course of these studies, the hexapeptide-bearing pins were reused through 26 repetitive ELISA cycles without noticeable loss of activity.

Subsequent screening of HDAg hexapeptides with four other high-titer anti-HD-positive human sera identified identical or closely positioned epitopes within the protein, as well as two additional antigenic domains. Antigenic domains of HDAg were defined as regions containing overlapping or contiguous hexapeptides found to be antigenic in screening with any of the five human anti-HD-positive serum samples (Fig. 3). Altogether, these domains included regions spanned by residues 2 to 7, 63 to 74, 86 to 91, 94 to 100, 159 to 172, 174 to 195, and 197 to 207 (Table 2). Although the carboxy-terminal 50 residues of the HDAg protein (residues 159 to 207) appeared immunodominant with each of the human sera tested in these assays, significant variation was evident between individual sera with respect to the degree to which certain hexapeptides were bound by antibody (Fig. 4). These results demonstrate the power of this approach to the fine-structure mapping of antigenic activity (11, 12). Among

A.

S R G A P G G G F V P S M Q	OD_{490}	Reactive Sera
S R G A P G	0.360	AC036
R G A P G G	0.348	AC036
A P G G G F	0.516	AC039
G F V P S M	0.407	AD062
F V P S M Q	0.859	AC036,AC039,AD062

B.

V P E S P F A R T G E G L D I R G S Q G F P	OD_{490}	Reactive Sera
V P E S P F	0.560	AC036,AC039,AF043,AD062
P E S P F A	0.892	AC036,AC039,AF043,AD062
E S P F A R	0.403	AC036,AC039,AD099
S P F A R T	0.642	AD062
A R T G E G	0.344	AD099
R T G E G L	0.225	AD099
E G L D I R	0.398	AD099
I R G S Q G	0.317	AD099
R G S Q G F	0.311	AC039
G S Q G F P	0.217	AC039

FIG. 3. Examples of two antigenic domains of HDAg determined by pin-based hexapeptide ELISAs. Antigenic domains were defined by overlapping or contiguous peptide sequences found to be reactive as hexapeptides when tested against any anti-HD-positive serum. Only reactive hexapeptides are shown. When the hexapeptide was reactive with more than one of the tested sera, the values shown represent the mean absorbance: (A) antigenic domain at residues 159 to 172; (B) antigenic domain at residues 174 to 195. See also Table 2.

TABLE 2. Antigenic domains of HDAg defined by overlapping hexapeptides with antigenic activity[a]

Serum sample	No. of hexapeptides within given antigenic domain of HDAg							
	1–7	63–74	86–92	94–100	121–128[b]	159–172	174–195	197–207
AD062				2		2	3	2
AF043							2	
AC036	1	2				3	3	2
AC039		3	2	1		2	5	3
AD099							5	
WC862	2	4			2			3
A008								
A073								

[a] Antigenic domains were defined by overlapping hexapeptides with antigenic activity determined by screening against the panel of human sera (Fig. 3). Shown are the number of hexapeptides within each domain that were determined to have antigenic activity with each individual serum specimen (absorbance, ≥0.2 for human sera or ≥0.5 for woodchuck serum).

[b] Recognized only by woodchuck serum.

the five anti-HD serum samples tested, AD099 demonstrated relatively low-level binding of antibodies to HDAg hexapeptides (maximum absorbance, 0.398, compared with 0.858 to 2.272 obtained with the other four serum samples). These results suggest that the sequential epitopes recognized by AD099 antibodies in HDAg immunoblots (Fig. 1, lane 5) may be poorly mimicked by synthetic peptides as short as six residues in length.

Mapping of HDAg epitopes recognized by the woodchuck. Hexapeptide ELISAs were carried out with serum from a woodchuck hepatitis virus carrier woodchuck (WC862) which was acutely superinfected with HDV. Serum was collected following HDAg clearance from serum and the appearance of anti-HD. The second antibody used in these hexapeptide ELISAs, rabbit anti-woodchuck immunoglobulin, generated higher background activity and required a different standard for positivity (absorbance, ≥0.5). The results of these tests (Fig. 5) indicated that the epitopes bound by woodchuck antibodies (residues 1 to 7, 63 to 71, 121 to 128, and 197 to 204) overlap with at least some of the antigenic domains recognized by humans (residues 2 to 7, 63 to 74, and 197 to 207). However, in contrast to the apparent immunodominance of the carboxy-terminal region of HDAg in humans, HDAg domains defined by hexapeptides spanning residues 1 to 7 and 63 to 71 were dominant in this one woodchuck. In addition, woodchuck antibodies bound also to hexapeptides spanning residues 121 to 128, a region not recognized by any of the human sera tested (Table 2). These results suggest significant interspecies differences in the recognition of HDAg epitopes, although additional sera will need to be tested to completely define these differences.

Comparison of hexapeptide ELISA with computer algorithm predictions. We analyzed the predicted amino acid sequence of HDAg for regions of relative hydrophilicity and hydrophobicity, using the method of Hopp and Woods (14), and values of relative hydrophobicity developed for individual amino acid side chains by Fauchère and Pliška (9). Although these two methods yield somewhat different predictions of the hydrophilic and hydrophobic domains of the protein, both suggest that the carboxy-terminal region (residues 145 to 214) of HDAg is less hydrophilic than the amino-terminal 144 residues (not shown). Neither analysis would have predicted antigenic domains occurring within residues 159 to 207 that appear dominant in hexapeptide ELISAs with human sera.

Similarly, secondary-structure predictions had little success in predicting these epitopes. Predictions of the second-

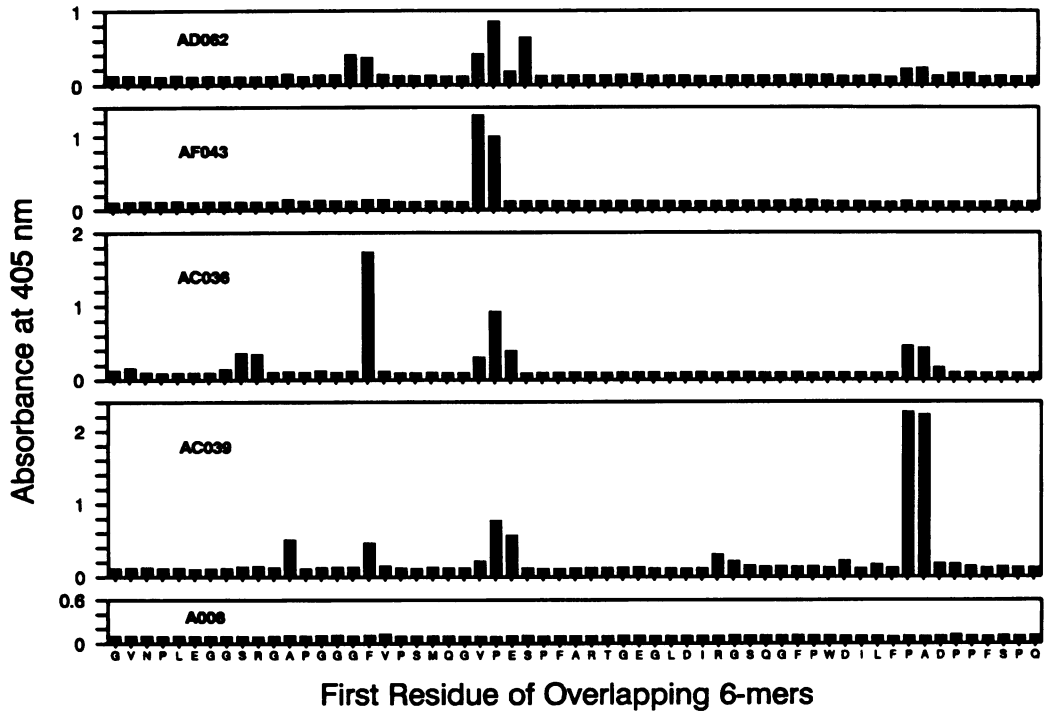

FIG. 4. Pin-based hexapeptide ELISA with hexapeptides spanning residues 151 to 214 of HDAg (carboxy-terminal 64 residues). A008 is a control serum, while the other four serum samples contain high titers of anti-HD (Table 1).

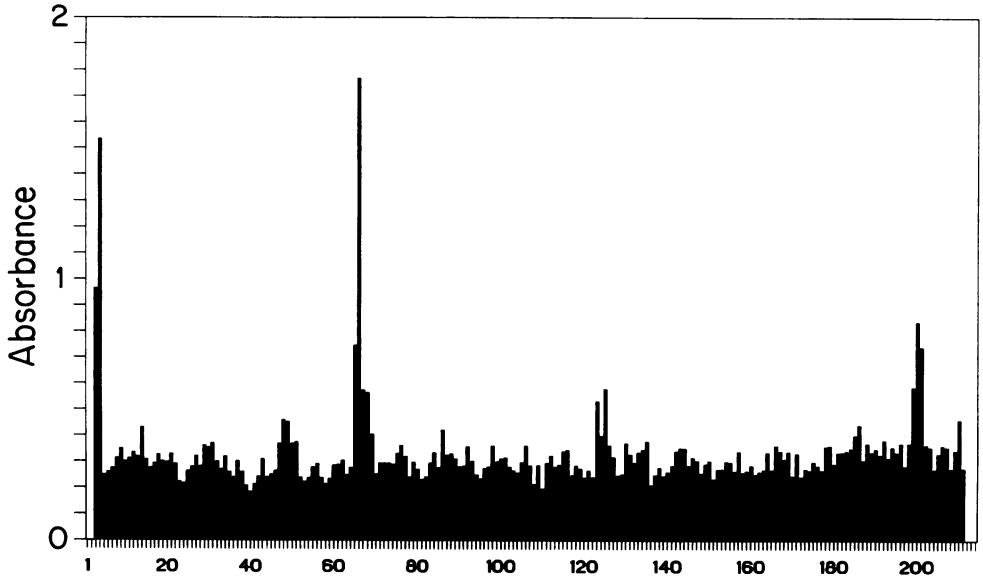

FIG. 5. Hexapeptide ELISA scan of HDAg with woodchuck serum WC862. Pins were considered positive when the absorbance was ≥0.500.

ary structure of HDAg made by the PEPTIDESTRUCTURE program (15) are shown in Fig. 6. For each hexapeptide, we cross-classified observed antigenic activity against predictions of hydrophilicity (seven-residue window), surface probability, flexibility, secondary structure, and antigenic index (15) generated by PEPTIDESTRUCTURE. Sensitivity, specificity, and positive and negative predictive values (maximum 51 and 70%, respectively) were calculated for each prediction rule (not shown). None of the methods were good predictors. Regardless of the prediction method, the kappa statistic, which measures chance-adjusted agreement, never exceeded 0.21, indicating poor agreement between predicted and observed antigenicity. Nonetheless, each of the antigenic domains shown in Table 2 contains or is immediately adjacent to turns in the HDAg structure predicted by the Chou-Fasman (6) or Garnier-Osguthorpe-

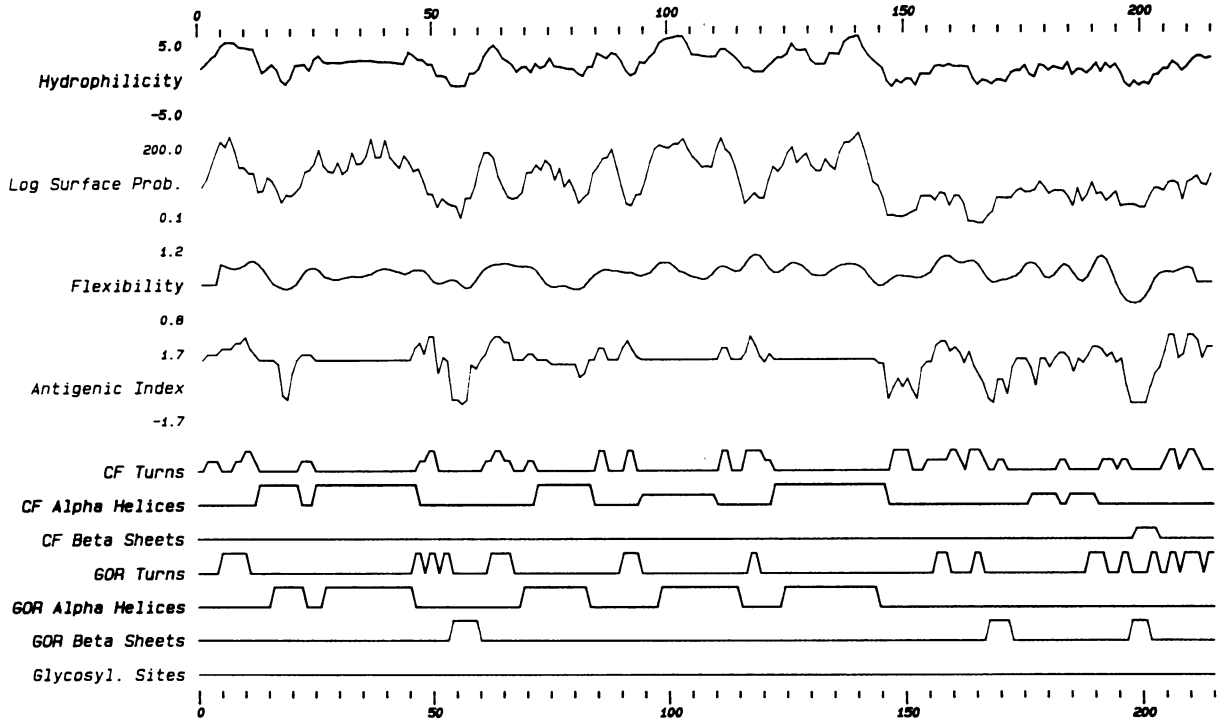

FIG. 6. Analysis of the predicted HDAg (19) secondary structure by the PEPTIDESTRUCTURE program (15). Shown is the PLOTSTRUCTURE program output detailing hydrophilicity (Kyte-Doolittle), surface probability (Emini), chain flexibility (Karplus-Schulz), antigenic index (Jameson-Wolf), and secondary structure by the methods of Chou-Fasman (CF) and Garnier-Osguthorpe-Robson (GOR).

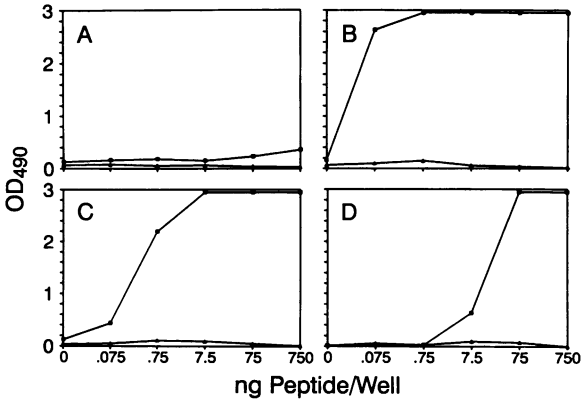

FIG. 7. Oligopeptide ELISA for anti-HD. An anti-HD-positive serum (AC036) and a negative serum (A008) were each tested at a dilution of 1:1,000 against decreasing quantities of synthetic oligopeptides applied to the wells of microdilution ELISA plates. (A) Peptide 2–17; (B) peptide 156–184; (C) peptide 167–184; (D) peptide 197–212.

Robson (10) method (Fig. 6). Notably, of a total of 17 proline residues within the HDAg protein, 13 occur within or immediately adjacent to hexapeptides reactive in ELISA. The possible influence of proline residues in two of the antigenic domains is evident in Fig. 3. In both instances in which there are two adjacent proline residues within the protein (residues 69 to 70 and 204 to 205), there was evidence of antigenic activity. Finally, although regions predicted to be amphipathic alpha-helices are generally considered predictive of T-cell and not B-cell determinants (20), it was of interest that five of nine predicted amphipathic segments (residues 117 to 120, 131 to 133, 150 to 159, 170 to 177, and 179 to 183) were adjacent to or overlapped with antigenic sites determined in hexapeptide ELISAs.

Microdilution oligopeptide ELISA for anti-HD. Based on the results of hexapeptide ELISAs with human sera, four oligopeptides representing HDAg residues 2 to 17, 156 to 184, 167 to 184, and 197 to 211 were synthesized in bulk and tested for antigenic activity by a microdilution ELISA. The results of assays with these bulk-synthesized peptides confirmed the validity of the pin-based hexapeptide ELISA screening tests (Fig. 7). Maximal antigenic activity was associated with the largest peptide, representing residues 156 to 184, which demonstrated substantial antigenic activity with as little as 75 pg of peptide applied in solution to ELISA plate wells (Fig. 7B). The 156 to 184 peptide contains an internal tripeptide sequence of three glycine residues (164 to 166), suggesting that it may comprise two distinct functional domains; thus, peptide 167-184 was synthesized to represent the carboxy domain of this peptide. Results shown in Fig. 7B and C suggest that domains on either side of the three glycine residues contribute to the antigenic activity of the 156–184 peptide. Peptide 167–184 was strongly antigenic, although less so than the 156–184 peptide. It should be noted, however, that the hexapeptide APGGGF (162 to 167) had significant antigenic activity in hexapeptide screening assays (Fig. 3A). Less antigenic activity was evident with a peptide representing residues 197 to 211, and only limited activity was found with peptide 2–17 (Fig. 7a and d). Although we did not assess the efficiency with which these peptides bound to the plastic microdilution plate, the degree of reactivity of each of the human sera with individual peptides was proportionate to the degree of binding of antibodies in pin-based hexapeptide ELISAs (Table 3). Serum AD099 did not react with any of the four bulk-synthesized peptides, consistent with the fact that its reactivity with hexapeptides suggested dominant (but relatively weak) reactivity in the region 180 to 193.

TABLE 3. Detection of anti-HD by synthetic peptide ELISA

Serum sample	OD_{490} at 1:100 serum dilution (endpoint titer)			
	2–17[a]	156–184	167–184	197–211
AD062	0.07 ($<10^2$)	$\geq$2.95 ($\geq10^6$)	$\geq$2.95 (10^5)	0.34 (10^2)
AF043	0.06 ($<10^2$)	$\geq$2.95 (10^5)	1.29 (10^3)	0.28 (10^2)
AC036	0.33 (10^2)	$\geq$2.95 ($\geq10^6$)	$\geq$2.95 ($\geq10^5$)	2.53 (10^2)
AC039	0.09 ($<10^2$)	$\geq$2.95 ($\geq10^6$)	$\geq$2.95 (10^5)	$\geq$2.95 (10^4)
AD099	0.09 ($<10^2$)	0.04 ($<10^2$)	0.05 ($<10^2$)	0.03 ($<10^2$)
A008	0.01 ($<10^2$)	0.02 ($<10^2$)	0.02 ($<10^2$)	0.02 ($<10^2$)
A073	0.01 ($<10^2$)	0.05 ($<10^2$)	0.03 ($<10^2$)	0.02 ($<10^2$)

DISCUSSION

Although the genomic and antigenomic RNAs of HDV potentially encode several additional proteins (17, 19, 26, 27), HDAg is the only viral protein known to be expressed during HDV infection in vivo. The function of this protein in the replicative cycle of HDV is not clear. HDAg may have a role in packaging of HDV RNA and assembly of the HDV particle. However, the nuclear location and apparent RNA-binding activity of HDAg suggest that it may provide additional functions necessary for the replication of viral RNA (5). That this is so is further suggested by the recent observation that expression of HDAg promotes the replication of HDV RNA following transfection of cell cultures with cloned HDV cDNA (16). While the HDAg domain involved in its putative RNA-binding activity is unknown, it seems likely that this function may be mediated by the highly basic amino half of the molecule. It is of interest that the major antigenic domains recognized by human antibodies were found in the less hydrophilic carboxy region of the protein. Reactivity of human sera with peptides representing residues 197 to 207 of HDAg provides strong evidence that the entire HDV protein is expressed in vivo, as these residues lie downstream of the amber stop codon found in some HDV cDNA clones at residue 195 (16, 28).

The approach we utilized for mapping the epitopes of HDAg involved the synthesis of 209 hexapeptides, overlapping each other by 5 residues and spanning the entire 214 residues that most likely constitute the $p27^{\delta}$ form of HDAg. These peptides were synthesized on polyethylene pins configured in a microdilution format, a method first described by Geysen et al. (11) that allows their direct and repeated use in ELISA to determine antibody binding activity. This method has previously been utilized to map epitopes of several proteins (11–13) and, based on our results, appears to be particularly useful when the epitopes of interest are sequential and easily detected in immunoblots of denatured antigen. The power of this procedure rests in the ease and economy with which many peptides can be synthesized and tested for antigenic activity. The analysis presented in this paper represents the results of >1,000 separate tests involving unique antibody-peptide combinations, many of which were repeated several times.

An obvious limitation of this epitope-mapping technique, however, is its inability to map epitopes that are conformationally defined. Whether such assembled epitopes contrib-

ute to the overall antigenicity of HDAg cannot be assessed from the studies reported here, but this possibility must be considered likely. The probability that HDAg has additional conformationally defined epitopes is increased by the fact that we have been unable to block effectively the binding of polyclonal human antibody to woodchuck liver-derived HDAg with excess quantities of the four peptides listed in Table 3 (J.-G. Wang and S. M. Lemon, unpublished data). Given the expectation that oligopeptide mapping will fail to define conformational epitopes, it is remarkable that the antigenic domains shown in Table 2 involve 88 (41%) of the 214 amino acids residues in the protein. This observation is consistent with the suggestion that most if not all of the accessible surface of a protein may be immunogenic in its native state (2).

A second and less obvious limitation of the pin-based oligopeptide approach to epitope mapping is related to the purity of the peptides synthesized on pins. As these peptides are not cleaved from the pins following their synthesis and undergo no physical purification steps, impurities due to unplanned side chain reactions or other irregularities in the synthesis accumulate with the addition of residues to the immobilized peptide. Thus, there are inherent limitations to the length of peptides that can be usefully synthesized in this fashion. While 6-mers are at the extreme minimum with respect to the limits on length of peptides that would be expected to preserve antigenic activity (12), it may be argued that screening by ELISA against hexapeptides provided a stringent approach capable of detecting short domains having the greatest affinity for relevant antibodies. Whether screening against larger peptides (8-mers or 10-mers, which are possible by this method) would have resulted in a significantly different picture of the epitopes of HDAg is unknown. This would appear to be unlikely, however, given the results of microdilution ELISAs with bulk-synthesized oligopeptides which were 15 to 29 residues in length (Table 3). These results, when analyzed quantitatively, were entirely what would have been predicted from the hexapeptide-screening ELISAs.

Consistent with its proposed role as a nucleic acid-binding protein, HDAg has been shown to be phosphorylated at multiple serine residues (5). The contribution of serine phosphorylation to the antigenicity of the molecule is unknown and was not explored in these studies with synthetic peptides. Lack of phosphorylation among serine residues in the synthetic hexapeptides is unlikely to have contributed to an inability to detect immunogenic domains, however, as the majority of the serine residues in HDAg occur in regions that were identified as contributing to epitopes. It is possible, however, that other posttranslational modifications to HDAg may affect the antigenicity of the native protein.

The data presented in Table 3 suggest the possibility that synthetic peptides might be useful diagnostic reagents that could be employed in new assays for anti-HD antibodies. Recent studies with the 156–184 peptide suggest that it is recognized by IgG antibody present in approximately 90% of anti-HD-positive sera and that it may provide the basis for an antibody test that is significantly more sensitive than existing competitive immunoassays (Wang and Lemon, unpublished data). Further studies of the immune response to this and other HDAg peptides will be of interest in determining whether there are differences in the fine structure of the B-cell response to this protein in acute and chronic HDV infections and in assessing the isotype-specific responses to this antigen.

In addition, it is known that previous HDV infection of HBV carrier chimpanzees results in at least partial protection against severe hepatitis following reexposure to HDV (23). The molecular basis of this immunity is not clear. However, inasmuch as HDAg is the only protein product known to be expressed by the HDV genome during infection in vivo, it is likely that this protection reflects the humoral or cellular immune response to HDAg. It is therefore intriguing to speculate that immunization with one or more of these antigenic HDAg peptides might provide a means of protection of HBV carriers from HDV superinfection. Given the existence of 200 million carriers of HBV worldwide, exploration of the protective efficacy of such immunogens for HDV would seem well worthwhile.

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