Immune Response to Synthetic Peptides of Hepatitis Delta Antigen

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Hepatitis delta antigen (HDAg) is the only viral protein known to be expressed during hepatitis delta virus (HDV) infection. Detection of antibody to HDAg (anti-HD) is the usual method for diagnosis of HDV infection since viremia lasts only a few weeks. In an effort to identify the major epitopes recognized by humans during natural infection, four oligopeptides including residues 2 to 17 (SP1), 155 to 172 (SP2), 168 to 182 (SP3), and 189 to 211 (SP4) of the HDAg molecule were synthesized and probed by enzyme-linked immunosorbent assay with a panel of 50 serum specimens from 45 patients suffering from either HDV-hepatitis B virus coinfections (n = 17) or HDV superinfections (n = 28). Sera from infected patients recognized these relatively short peptides. Peptide SP2 was the most antigenic: 71% of serum specimens reacted. Antibody to SP2 was also the commonest in sera taken early in the course of the disease. Peptide SP2 corresponds to one of the two regions which is highly conserved between different isolates. Among the 63 serum specimens which scored anti-HD positive by a commercial assay, all but 3 reacted to at least one of the peptides (95% agreement). Peptide assays appeared to be significantly more sensitive than the commercial assay with native HDAg early in the course of HDV infection since 14 of 17 (82%) serum specimens which scored anti-HD negative in the commercial assay reacted to one or more peptides. All serum specimens giving one or more positive results with the various peptides were confirmed as being HDV positive by an inhibition assay with free peptide in solution. The immune response to HDAg peptides varied greatly between individuals. No specific reactivity profile could be assigned to those with either HDV-hepatitis B virus coinfections or HDV superinfections. Overall, HDAg peptides appeared to be convenient reagents in addition to native antigen for the development of new and improved diagnostic tests for HDV infection.

Infection with hepatitis delta virus (HDV) is a major cause of severe acute hepatitis and progressive chronic liver disease (12, 20). HDV is a small defective RNA virus which is dependent on hepatitis B virus (HBV) for essential helper functions. The HDV virion consists of a 1.7-kb single-stranded circular RNA genome, a delta antigen, and a lipid envelope containing hepatitis B surface antigen (HBsAg) provided by its helper virus (21). HDV infection can be acquired only in the presence of HBV either by coinfection with both viruses or by HDV superinfection of an HBsAg carrier.

The HDV genome may potentially encode for up to five different proteins of significant length. To date, hepatitis delta antigen (HDAg) is the only protein known to be expressed during in vitro and in vivo infections and corresponds to one of the open reading frames of the antigenomic strand RNA (5, 26). It has been detected in the virions and nuclei of infected cells in two different forms, one large form of 214 amino acids (aa) and another smaller form of 195 aa resulting from an amber mutation (2, 19, 26). HDAg is a highly basic phosphoprotein which can bind to RNA molecules (5, 15). It seems that only the 195-aa HDAg molecule is able to initiate genomic replication, while the 214-aa HDAg acts as an inhibitor of the trans activity of the 195-aa HDAg (6, 11, 13, 23).

During hepatitis caused by HDV, patients develop a specific humoral response to HDAg (anti-HD). Detection of anti-HD is the only practical approach to diagnosis of the infection since viremia lasts only a few weeks in most immunocompetent patients (8). Dissection of the antigenic structure of HDAg and the associated immune response is important for understanding the pathogenicity of HDV and its eventual prevention. In the study described here, our aims were to analyze the immune response during natural HDV infection and to identify determinants that could be of use for diagnosis. We studied the abilities of antibodies in sera from 45 HDV-infected patients to bind to four synthetic oligopeptides of 15 to 23 residues in length corresponding to putative epitopes of HDAg (24).

MATERIALS AND METHODS

Human sera. Sera were collected from patients with acute or chronic hepatitis hospitalized in the Tours University Hospital. HBV and HDV infections were diagnosed by using commercially available enzyme-linked immunosorbent assays (ELISAs). Tests for anti-HD and HDAg were carried out with the Hepanostika enzyme immunoassay (EIA; Organon Teknika, Boxtel, The Netherlands), tests for anti-HD immunoglobulin M (IgM) were carried out with Eti-Delta-IgMK (Sorin Biomedica, Saluggia, Italy), tests for HBsAg were carried out with EIA Hepanostika HBsAg (Organon Teknika), tests for antibodies to HBsAg (anti-HBs) were done with AUSAB-EIA (Abbott, North Chicago, Ill.), tests for antibodies to hepatitis B core antigen (anti-HBc) were done with Eti-AB-Corek (Sorin Biomedica), and tests for anti-HBc IgM were carried out with Corzyme-M (Abbott). Two groups of serum specimens were selected and tested for the presence of antibody to the four peptides. Group 1 (HDV
TABLE 1. HBV and HDV markers of sera from HDV-infected patients

<table>
<thead>
<tr>
<th>Group</th>
<th>Stage of infection</th>
<th>No. of specimens</th>
<th>Presence of marker</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>HBsAg</td>
</tr>
<tr>
<td>HDV coinfection</td>
<td>Early</td>
<td>13</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Late</td>
<td>15</td>
<td>-</td>
</tr>
<tr>
<td>HDV superinfection</td>
<td>Early</td>
<td>7</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Late</td>
<td>41</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Late with persistent HDAg</td>
<td>4</td>
<td>+</td>
</tr>
</tbody>
</table>

* Except for three serum specimens which were negative but drawn in the first few weeks after onset.

b Except for one serum specimen which was positive but drawn in the fourth week after onset.

c Except for two serum specimens which were positive but drawn in the first few weeks after onset.

positive) consisted of 80 serum specimens from 45 patients coinfected or superinfected with HDV. They were further divided into the following five classes according to their serological status and medical records: sera from patients with early- or late-stage HDV-HBV coinfections, sera from patients with early- or late-stage superinfections, and sera from patients with superinfections with persistent HDAg (Table 1). Specific criteria for an HDV-HBV coinfection versus an HDV superinfection were the presence of anti-HBc IgM and HBsAg in the first stage of the infection followed later by the disappearance of these markers in a coinfection, whereas in a superinfection, HBsAg was present and anti-HBc IgM was absent (Table 1). Sera drawn within 1 to 2 months of the onset of HDV infection (early stage of infection) were all positive for HDAg except for three serum specimens drawn a few days after the disappearance of the antigen. Sera from the late stage of infection were all HDAg negative, with the exception of four serum specimens which were drawn late in the course of the infection from patients with persistent HD antigenemia (22). The commercial assay detected anti-HD antibody in 63 (16 from patients with coinfections and 47 from patients with superinfections) of the 80 serum specimens.

Group 2 (HDV negative, HBV negative) consisted of 93 control serum specimens which were negative for HBsAg, anti-HBc, anti-HBc IgM, and anti-HBs as well as HDAg and anti-HD.

**Synthetic peptides.** Synthetic peptides corresponded to parts of the amino acid sequence of HDAg as deduced from the nucleotide sequence reported by Wang et al. (25). Two highly conserved regions were found in the HDAg sequence (residues 84 to 111 and 150 to 169) (7). The first of these two regions has recently been shown to be the RNA-binding domain of HDAg which binds specifically to HDV RNA (15). The biological function of the second region is not defined. B-cell epitopes were predicted by using a computer program, which was kindly provided by J.-D. Guitton (Rhone-Poulenc, Vitry, France), and the algorithm of Parker et al. (18) on the basis of the following three antigenicity factors: hydrophilicity, accessibility, and flexibility. Eight sequences were predicted to be antigenic: those from residues 3 to 12, 29 to 43, 59 to 66, 83 to 89, 95 to 105, 123 to 130, 134 to 142, and 156 to 165 (Fig. 1). Further indications for potential epitopes were drawn from the literature. Bergmann et al. (1) reported human sera directed against the sequences at positions 1 to 18, 35 to 52, 51 to 69, 65 to 81, 90 to 107, 120 to 138, and 173 to 191, and Wang et al. (24) reported sera directed against the sequences at positions 1 to 7, 63 to 74, 86 to 92, 94 to 100, 159 to 172, 174 to 195, and 197 to 207. Four sequences were finally selected. They overlap two of the predicted sequences (peptide SP1, residues 2 to 17; peptide SP2, residues 155 to 172) and two of the epitopes identified by Wang et al. (24) and Bergmann et al. (1) (peptide SP3, residues 168 to 182; peptide SP4, residues 189 to 211). The last peptide was specifically chosen because it covered the
TABLE 2. Peptide sequences and characteristics

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Corresponding sequence (residue no.)</th>
<th>Predicted antigenicity</th>
<th>Overlapping peptides tested by:</th>
<th>Characteristic</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP1</td>
<td>2–17</td>
<td>Yes</td>
<td>Yes</td>
<td>N-terminal extremity</td>
</tr>
<tr>
<td>SP2</td>
<td>155–172</td>
<td>Yes</td>
<td>No</td>
<td>Highly conserved sequence</td>
</tr>
<tr>
<td>SP3</td>
<td>168–182</td>
<td>No</td>
<td>Yes</td>
<td>C-terminal extremity of small HDAg</td>
</tr>
<tr>
<td>SP4</td>
<td>189–211</td>
<td>No</td>
<td>Yes</td>
<td>Overlapping ends of both HDAg forms</td>
</tr>
</tbody>
</table>

Peptides were synthesized with an automated peptide synthesizer (431A; Applied Biosystems) by the solid-phase procedure developed by Merrifield (16), with 9-fluorenylmethoxycarbonyl-protected amino acids and p-hydroxymercaptohexyloxycarbonyl resin. After synthesis, the resin support and the side chain-protecting groups were removed by treatment with trifluoroacetic acid in the presence of scavengers. Peptides were purified by reverse-phase chromatography with C8 columns (Aquapore octyl; 20 μm; 100 by 10 mm; Applied Biosystems). The purities of the preparations were confirmed by the presence of a single sharp peak on high-pressure liquid chromatographic analysis of C8 columns (Aquapore octyl; RP-300; 30.0 nm; 4.6 by 220 mm; Applied Biosystems) and amino acid analysis (9). Peptides were conjugated to bovine serum albumin (BSA) with carbodiimide. The coupling yield of each peptide was determined by analyzing the amino acid composition of the coupled peptide; 4, 7, 10, and 5 molecules of peptides SP1, SP2, SP3, and SP4 were bound per molecule of BSA, respectively.

Peptide ELISA for anti-HD. To optimize the peptide ELISA for anti-HD, we carried out multiple experiments with different plates, coating buffers, and free or coupled peptides. The best antigenicity results were obtained with peptides coupled to BSA. Peptides coupled to BSA were incubated in the wells of flat-bottom ELISA polystyrene plates (CML-CEB, Neumours, France) at a concentration of 10 μg/ml in 0.05 M bicarbonate buffer (pH 9.6), for 20 h at 4°C (200 μl per well). Plates were washed three times with 0.01 M phosphate-buffered saline (PBS)-0.15 M NaCl (pH 7.4) containing 0.5% Tween 20 and were blocked by the addition of 300 μl of PBS containing 2% newborn calf serum for 45 min at 37°C. After three washings, 150 μl of the serum specimen to be tested, which was diluted 1:10 in the test buffer 0.05 M PBS and 0.75 M NaCl (pH 7.4) containing 5% newborn calf serum, 5% BSA, and 0.5% Tween 20, was then added and the samples were incubated for 30 min at room temperature. Following six washings, 100 μl of horseradish peroxidase-conjugated goat anti-human F(ab')2 (Tago, Burlingame, Calif.) diluted 1:10,000 in the same buffer was added. The plates were incubated for 30 min at room temperature and washed six times, and a mixture of hydrogen peroxide–o-phenylenediamine was added at room temperature in the dark. Color development was stopped with 2 N H2SO4, and the A492 was read.

A control assay with only BSA coated onto the solid phase was performed to assess assay specificity. A cutoff value was calculated for each peptide by using the results obtained with the 93 HBV-negative, HDV-negative sera (negative controls). The mean optical density (OD) values were 0.076, 0.021, 0.065, and 0.027 for the tests with peptides SP1, SP2, SP3 and SP4, respectively. The mean OD value plus 3 standard deviations was chosen as the cutoff value, i.e., 0.242, 0.106, 0.282, and 0.141 for the assays with SP1, SP2, SP3, and SP4, respectively.

Inhibition procedure. All sera that gave one or more positive results with any of the different peptides were further tested for their ability to bind in the presence of increasing concentrations of free peptide. Briefly, after the blocking step of the peptide ELISA, 50 μl of a series of concentrations (0.01 to 8 μg per well) of each peptide was added to the wells of the corresponding assay. A total of 50 μl of serum sample diluted 1:10 in the test buffer was then incubated at room temperature in the presence of free peptide for 30 min. The subsequent steps of the assay were as described above.

RESULTS

Immune response to HDAg peptides. All four peptides were recognized by at least 1 of the 80 tested serum specimens: 38, 71, 45, and 63% of the serum specimens recognized peptide SP1, SP2, SP3, or SP4, respectively. Of the 36 serum specimens that reacted with peptide SP3, 32 serum speci-

![Graph A](http://jcm.asm.org/)

![Graph B](http://jcm.asm.org/)
mals also recognized peptide SP2. Absorbance values obtained with peptide SP3 were significantly higher than those obtained with peptide SP1 ($P < 10^{-5}$), SP2 ($P < 10^{-5}$), or SP4 ($P < 10^{-5}$) (Fig. 2A and B).

All but 3 of the 63 serum specimens (16 from patients with HDV-HBV coinfections and 47 from patients with HDV superinfections) which scored anti-HD positive by the Hepanostika assay were also positive with one (n = 13), two (n = 21), three (n = 12), or four (n = 14) of the peptides. Only 6 of the 80 tested serum specimens were negative in our assay; 3 were early serum specimens from coinfected patients which scored anti-HD negative by the Hepanostika assay, 1 was a late serum specimen from a coinfected patient, and 2 were late serum specimens from superinfected patients which scored anti-HD positive by the Hepanostika assay.

Among the 74 serum specimens which recognized one or more peptides, 30 (41%) reacted to SP1, 57 (77%) reacted to SP2, 36 (49%) reacted to SP3, and 50 (68%) reacted to SP4 (Table 3). The immune responses to HDAG peptides of the different groups of HDV patients were heterogeneous.

Sera from patients with HDV-HBV coinfections reacted more frequently to peptide SP2 (83%) than to SP1 (25%; $P < 0.0001$), SP3 (29%; $P < 0.001$), or SP4 (46%; $P < 0.01$). Sera from patients with HDV superinfections also reacted more frequently to peptides SP2 (74%) and SP4 (78%) than to peptides SP1 (48%) and SP3 (58%), but the difference was not significant.

Early sera reacted more frequently to SP2 (88%) than to SP1 (24%; $P < 0.001$), SP3 (35%; $P < 0.01$), and SP4 (53%; $P < 0.05$). Late sera also reacted more frequently to peptides SP2 (74%) and SP4 (72%) than to peptides SP1 (44%; $P < 0.01$) and SP3 (53%; $P < 0.05$).

Only 3 of the 20 early serum specimens scored anti-HD positive by the commercial assay. These 3 serum specimens and 14 of the 17 anti-HD-negative early serum specimens reacted to one or more peptides; 20, 75, 30, and 45% reacted with peptide SP1, SP2, SP3, or SP4, respectively. Early in the course of HDV infection, the sensitivity of the peptide ELISA (for all four peptides cumulatively) was thus 85% versus 15% for the commercial assay ($P < 0.001$). Diagnosis of HDV infection could be established with the peptide assays earlier in the course of infection than with the commercial assay in eight cases. However, it should be noted that four of these cases would have been detected by a commercial immunocapture IgM assay.

The kinetics of the antibodies to the different peptides at early and late stages of the disease could be studied in serial samples from five patients (four with coinfections and one with a superinfection). Antibody to peptide SP2 was the first to appear for three of the four patients with coinfections. The reactivities of the sera to peptide SP2 increased prior to the anti-HD seroconversion, as defined by the commercial assay (Fig. 3A). In the patients with superinfections, two bouts of HD antigenemia were observed. Antibody to peptide SP2 was the first to appear early in the course of the infection. Reactivities to peptides SP1 and SP2 increased during the second bout of HD antigenemia (Fig. 3B). The kinetics of antibody to the various peptides could also be studied in serial samples from four other patients with late-stage superinfections. In one patient who was negative for anti-HD IgM,
indicating either a less active disease or elimination of HDV (3), antibody titers to peptides SP2, SP3, and SP4 declined over a period of 12 months. In the three other patients who were anti-HD IgM positive (indicating a chronic HDV infection), a high antibody titer plateau was observed for peptide SP4 in two patients and for SP3 in one patient.

Inhibition assays. The specificities of the sera for the various peptides were determined by an inhibition assay with a series of concentrations of peptide (not conjugated to BSA) to block the reactivity of sera in the homologous peptide ELISA. Reactivity to each peptide could be specifically reduced by at least 50% by the addition of 8.0 μg of the corresponding peptide (Fig. 4).

**DISCUSSION**

The HDV genome appears to produce only one protein, HDAg, during its cellular cycle (26). HDAg plays an essential role both in the regulation of HDV replication and in the interference of HDV with the HBV, its helper virus (28). HDAg is immunogenic during natural infection in humans. Testing for antibody to HDAg has become the standard laboratory diagnostic approach. Various tests for anti-HD have been developed, and some are available commercially. The results obtained with these tests may differ (8), indicating that the immune response to HDAg among infected patients is heterogeneous and that a better knowledge of HDAg epitope specificities is badly needed.

Synthetic peptides have proved to be very valuable tools for the study of protein structure and antigenic determinants and the analysis of the fine immune responses of patients to various antigens (14, 17). Using HDV synthetic peptides and five serum specimens from HDV-infected patients, Wang et al. (24) have shown that essential epitopes for recognition by the immune system were located within sequences corresponding to residues 1 to 7, 63 to 100, and 159 to 207 of HDAg. In their study, maximal antigenic activity was associated with a peptide representing residues 156 to 184. That work and further epitope prediction analysis led us to synthesize four peptides scattered along the HDAg sequence and covering regions that were predicted to be either immunogenic or highly conserved. These peptides were used to set up an EIA and screen a panel of serum specimens from 45 patients suffering from either HDV-HBV coinfections or HDV superinfections.

We confirmed that relatively short peptides could be recognized by sera from infected patients but that none of the four peptides elicited antibody in every positive patient. Maximal antigenic activity was observed with peptide SP2 (aa 155 to 172), to which 71% of sera reacted. Antibody to SP2 was also the commonest (75%) in sera drawn during the first 2 months following the onset of HDV infection. This peptide corresponds to one of the two regions (aa 84 to 111 and aa 150 to 169) found to be highly conserved between different isolates (7). Although predicted to be antigenic by sequence analysis, peptide SP1 (aa 2 to 17) was recognized by only 38% of the tested serum specimens. These results agreed with those obtained by Wang et al. (24) with a small number of serum specimens. Discrepancies between pre
dicted peptide antigenicity and experimental results with human sera illustrate the limitations of current algorithms. It is also possible that initial binding between antibody and virus promotes conformational changes of the native antigen that mask previously exposed structures (10). All four peptides were coupled to BSA to improve their antigenicities in the solid-phase immunoassay. The coupling yield of peptide SP1 was only four peptide molecules per albumin molecule. This relatively low yield may also explain in part the poor results obtained with this peptide. Of 36 serum specimens that reacted with peptide SP3 (aa 168 to 182), 32 were also reacted positively with peptide SP2. The 5-aa overlap between these two sequences may explain this result. The absorbance values (hence binding) obtained with peptide SP3 were significantly greater than those observed with the three other peptides. It is possible that when this peptide is fixed to the solid phase it remains in a conformation closest to that in the native protein and underlines the good accessibility of this peptide, which is located in the carboxy-terminal region of the short 195-aa HDAg molecule.

Of 63 serum specimens (16 from patients with HDV-HBV coinfections and 47 from patients with HDV superinfections) which were anti-HD positive with a commercial assay, all but 3 reacted to at least one of the peptides, a 95% agreement. The three serum specimens that were negative with the peptide ELISA were samples from patients at the late stage of infection (one from a patient with a coinfection and two from patients with superinfections). These discrepancies may correspond either to a false-positive reaction of the anti-HD Hepanostika assay or to sera that reacted to minor or conformational antigenic determinants. On the other hand, the peptide ELISA appeared to be significantly more sensitive than the anti-HD Hepanostika assay early in the course of HDV infection, since 14 of 17 (82%) serum specimens which were anti-HD negative with the commercial assay reacted to one or more peptides. Anti-HD Hepanostika is an indirect assay in which anti-HD from the serum specimen competes with anti-HD of the solid phase to bind to the added HDAg. This type of test can be unsatisfactory when it is used to test sera from patients with early-stage HDV infections, because then, sera very often contain both antibody and free antigen that are accessible without detergent treatment (4, 8, 22). Indeed, 15 of these 17 serum specimens were also HDAg positive. Our peptide ELISA was a direct assay that was less likely to be affected by such interference. The last two serum specimens which were anti-HD and HDAg negative with commercial assays but which scored positive in the peptide ELISA were collected during the seroconversion phase from two patients with HDV coinfections. These patients later scored anti-HD positive with the commercial assay.

The immune response to HDAg peptides differed between patients suffering from HDV-HBV coinfection or HDV superinfection and also varied according to the delay between the time of sampling and the onset of hepatitis. Antibodies to peptides SP1 and SP3 appeared significantly less frequently in sera from patients with early-stage coinfections than in sera from patients with early-stage superinfections and HDV-HBV coinfections. The prevalence of antibody to the different peptides appeared higher in sera from patients with late-stage superinfections than in those from patients with coinfections, but owing to the size of the panel, the difference was not statistically significant. Antibody titers to the various peptides declined during the convalescent period for one patient with a coinfection who was followed for 1 year. Similar findings were found for serum samples from a patient with a late-stage superinfection who was negative for anti-HD IgM, indicating a less active disease or the elimination of HDV (3). However, in three patients with superinfections who were positive for anti-HD IgM, sustained titers of antibody to various peptides in late-stage sera appeared to correlate with a profile usually considered to indicate active HDV replication.

Despite the great heterogeneity of serological profile observed between patients, synthetic HDAg peptides appear to be useful alternative reagents to native antigen in the development of new assays. A judicious combination of antibodies to peptides can deduced from our results, which showed that 72 of 74 (97%) serum specimens could be detected by the association of peptides SP2 and SP4 compared with 63 by the association SP1 and SP2 (85%; $P < 0.01$), 47 by SP1 and SP3 (64%; $P < 10^{-5}$), 57 by SP1 and SP4 (77%; $P < 0.001$), 61 by SP2 and SP3 (82%; $P < 0.01$), and 59 by SP3 and SP4 (80%; $P < 0.001$) (Table 4). The addition of a third peptide would not add a significant gain. To investigate this, 5 μg each of peptide SP2 and SP4 per ml was coated onto a single well and tested against 20 serum specimens. All serum specimens gave a positive result under these conditions, including 8 serum specimens that reacted only with SP2 or SP4 (seven and one serum specimen, respectively).

Peptide SP2 gives improved sensitivity in the early phase of infection and may prove very valuable for diagnosis, especially during the gap in time that usually follows the disappearance of HDAg.
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