Immune Response to Hepatitis A Virus Capsid Proteins after Infection

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This study was undertaken to determine the immune response of humans to viral capsid polypeptides of hepatitis A virus (HAV) after natural infection, which is very important for vaccine development. Antiviral capsids in 73 serum samples from patients with acute and chronic HAV infections were analyzed by immunoblotting against individual HAV capsid polypeptides (VP1, VP2, VP3, and VP4) by using a cell culture-based HAV antigen. For reference, total anti-HAV immunoglobulin G (IgG) and anti-HAV IgM were also determined by radioimmunoassay. As a result, a dominant immune response against VP1 (98% IgG, 94% IgM) was found in the acute phase. However, many other sera also reacted with VP0 (88% IgG; 35% IgM) and VP3 (81% IgG and 29% IgM). In contrast to the acute phase, anti-VP1, anti-VP0, and anti-VP3 IgG antibodies against all three viral proteins (29, 29, and 73%, respectively), especially those against VP3, were found years after onset of HAV disease and over long periods in the sera of hepatitis patients. These results suggest that antibodies for capsid polypeptides are present over an extended period in the sera of HAV-infected patients. They are likely of importance in maintaining long-term immunity.

Hepatitis caused by hepatitis A virus (HAV) is a common disease affecting humans and has a high incidence throughout the world. Recently, vaccines against the infection have become commercially available in certain areas of the world (1). Characterization of the HAV antibody requires quantitative description of immune protection against the infection and is of great importance in the practice of preventive medicine.

A better understanding of the immune response, antibody duration, and the quality of the anti-HAV antibody response in naturally infected individuals is essential for vaccine designers and producers. This knowledge could contribute to better evaluation of the immunogenicity and efficacy of vaccines and also shed light on the natural history of HAV infection (6). In particular, long-term follow-up of naturally infected patients is valuable not only for assessment of the duration of the immune response but also for determination of whether or not seroconversion with this intact immune memory indicates long-lasting protection from this disease.

The HAV molecule has a 7.5-kb single-stranded RNA genome. It has plus-strand polarity and contains a poly(A) region at the 3' terminus and a covalent, small oligopeptide (VPG) at the 5' end. Mature virus particles have cross-sectional diameters of approximately 27 nm. Three major capsid polypeptides with molecular masses of 30 to 33 (VP1), 24 to 27 (VP2), and 23 to 29 (VP3) kDa have been identified. The exact molecular weight of the small polypeptide (VP4) has not been determined (4). The epitopes of the capsid proteins were suggested to be very stable in long-term cultivation (21, 25).

HAV infection is diagnosed by detection of immunoglobulin M (IgM) or IgG antibodies to the capsid proteins (2, 23, 24). In general, these capsid antibodies are thought to be protective after a viral challenge. Nevertheless, linear or denatured capsid peptides are proposed to be poorly immunogenic in comparison with the native protein from which they have been derived. They are largely incapable of accurately representing their cognate counterparts, which are often conformationally restrained. This concept is still not experimentally proven and remains to be accurately defined.

Little experimental information on the acute-phase and long-term immune responses to capsid proteins after HAV infection is available (10, 13, 14, 18). Although purified HAV capsid polypeptides are thought to be, at least in certain cases, weak immunogens, VP1 has been identified as a major surface protein possessing a neutralizing immunogenic epitope. More recent studies have shown that neutralizing antibodies can be induced in rats after immunization with individually purified viral protein VP1, VP2, or VP3. Viral VP1 induced the strongest antibody response (24). Furthermore, Ping et al. (18) described an immunodominant neutralization site located on HAV capsid protein VP3. Cumulatively, it is of special interest to determine whether or not the sera of acute- and convalescent-phase patients contain antibodies to these HAV structural proteins.

This report describes experiments in which immunoblotting was used to characterize antibodies to HAV capsid proteins present in acute- and late-convalescent-phase serum samples from patients with hepatitis A. By use of a radioimmunoassay (RIA) and isotype-specific immunoblotting, acute-phase sera of hepatitis patients, as well as sera drawn several years after onset of the disease, were tested concurrently for anti-HAV IgM and total anti-HAV IgG immune responses.

MATERIALS AND METHODS

Serum samples. Anti-HAV antibody-positive serum samples from 31 patients with acute HAV infection, drawn between 0 and 67 days after the onset of icterus, were selected for this study. Long-term follow-up was carried out by studying the sera of eight patients. In each case, 2 to 14 serum samples were drawn on different occasions following the acute phase and during convalescence (between 6 months and 10 years). For comparison, 10 negative controls comprising serum samples from anti-HAV antibody-negative persons with no history of hepatitis A were included in the study.

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of HAV infection were used for the whole study. All of the above-mentioned serum samples were drawn from patients who were 25 to 45 years old.

**Isolation and concentration of HAV from cell culture.** HAV strain HFS/GBM was isolated and adapted to human diploid fibroblast cells as described earlier (6, 12). Fibroblast cells were cultured in Nunc cell factories (Nunc, Copenhagen, Denmark) and incubated in Earle’s minimum essential medium-supplemented medium with 4% fetal calf serum (GIBCO, Karlsruhe, Federal Republic of Germany) at 37°C. Dense fibroblast layers were infected with an HAV strain HFS/GBM seed pool. Four weeks later, the cells were treated twice with a lysis buffer (10 mM Tris HCl [pH 7.5], 1.5 mM NaCl, 1.5 mM MgCl₂, 0.1% Nonidet P-40) and cell debris was pelleted by low-speed centrifugation. The supernatant containing the HAV was dialyzed against phosphate-buffered saline and concentrated by high-speed centrifugation. These materials were further purified by cesium chloride equilibrium gradient centrifugation. A purified HAV density range (1.23 to 1.35 g/ml) was pooled and tested by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

**Immunoblot analysis.** Proteins in the HAV preparations were separated by SDS-PAGE in a discontinuous buffer system (15). The resolving gel contained 12.5% acrylamide, and the stacking gel contained 5% acrylamide. Electrophoresis was carried out at 80 V for 2 h (Electrophoresis Unit; Pharmacia LKB, Freiburg, Federal Republic of Germany). The separated proteins were then transferred to nitrocellulose (0.2-μm pore size; BA 83; Schleicher & Schüll, Dassel, Federal Republic of Germany) at 250 mA and 5°C overnight plus 1 h at 500 mA. The blot was then equilibrated in a solution of 10% nonfat milk in phosphate-buffered saline for 1 h at room temperature. The nitrocellulose was then cut into strips, and each strip was incubated overnight with either human anti-HAV antibody-positive serum or human anti-HAV antibody-negative serum. After washing, the second incubation phase was carried out over a 1-h period at room temperature with biotinylated anti-human IgG or IgM (1:1,000; Dianova, Hamburg, Federal Republic of Germany). After three rinsings, the membrane was made visible by using a streptavidin-conjugated peroxidase complex (1:200; Dakopatts, Copenhagen, Denmark) buffer with diaminobenzidine and H₂O₂.

**Detection of specific antibodies to HAV capsid proteins.** Immunoblotting experiments with HAV structural proteins that were probed with guinea pig sera specific for VP1, VP2, VP3, and VP4 have been previously documented (11). These synthetic peptides were a gift of D. Sangar (Wellcome Biotech). They were produced by coupling synthetic peptides to bovine serum albumin and then used to raise antibodies in guinea pigs. These antibodies were afterwards purified by affinity chromatography as described elsewhere (16). In our study, these anti-capsid sera were introduced into all immunoblotting assays at a dilution of 1:5,000.

Under these conditions, the estimated detection limit of this immunoassay was approximately 500 pg as determined with serial dilutions of HAV antigen (data not shown). In this study, however, to amplify the staining signal, we used 100 ng of the purified HAV in a single well (per lane) as a substrate for binding of the antibodies (from patients) with the separated capsids on the immunoblot. With this antigen assay, we classified the titers of anti-HAV total antibodies and IgM antibodies, both of which included the antibodies for structural proteins VP1 (anti-HAV VP1), VP0 (anti-HAV VP2), VP3 (anti-HAV VP3), and VP3 (anti-HAV VP4), in these serum samples.

Serum samples from one HAV-infected patient were chosen to demonstrate the efficacy of this method. The sera were incubated with the separated antigen immobilized on the blot and treated as previously described. The reactivity of anti-HAV IgG and IgM to VP1 and VP3 was determined.

**RIA for detection of anti-HAV IgG and IgM antibodies.** Anti-HAV antibody detection was done by a competition RIA, whereas anti-HAV IgM antibodies were detected by a modification of the anti-IgM technique described in an earlier report (8). The results were converted to international units by comparing them with a reference curve generated with the World Health Organization reference globulin. Seroconversion for HAV was considered positive at an anti-HAV IgG and IgM titers, with each sample presented as the result of two or more tests.

**RESULTS**

**Acute-phase and long-term anti-HAV total antibodies and anti-HAV IgM antibodies in HAV-infected sera.** The anti-HAV total-antibody titers and anti-HAV IgM antibody titers of our patients, as determined by RIA, are summarized in Fig. 1. Our results show that much lower anti-HAV antibody titers were observed during the initial phase of icterus (3 to 21 IU) during HAV infection. After 40 to 59 days of icterus, most of our samples had relatively higher counts of anti-HAV antibodies (298 to 1,500 IU). By contrast, many of the sera of acute-phase patients were marked by higher titers of anti-HAV IgM antibodies (1:50,000 to 1:2,250,000), especially the serum samples collected 11 days after the onset of icterus.

We further investigated the long-term change in the anti-HAV antibody titer during and after HAV infection. The anti-HAV antibody titers in the sera of eight patients were determined and plotted as illustrated in Fig. 2. For each patient, 2 to 14 serum samples were collected on different occasions after the acute phase, as well as during convalescence (from 6 months to 10 years). Our results show that four patients had maximum titers of anti-HAV antibodies after 71 and 513 days of icterus. In the long-term follow-up of one patient with a 10-year history, the anti-HAV antibody titer decreased continuously after 513 days. A titer of 31 IU of anti-HAV antibodies was detected in the serum of this patient even after 10 years. However, no IgM antibodies were detected 13 to 46 days after icterus, except in one serum sample after day 87.

**Purified HAV capsid proteins.** To provide antigens for this study, HAV molecules were purified by CsCl gradient ultracentrifugation and then partially characterized by an immunoblotting experiment. The antigenicity profile of the purified HAV after separation with a CsCl gradient ultracentrifugation is shown in Fig. 3A. Antigenicity was quantitated with a polyclonal anti-HAV serum. Antigenicity peaked in the fractions with CsCl densities of 1.23 to 1.33 g/cm³. These fractions were collected and separated by SDS-PAGE (Fig. 3B). The results demonstrated that most of the viral proteins VP1, VP0, and VP3 are concentrated. No nonstructural proteins were detected. This result agrees with a previous observation that capsid proteins are produced in greater abundance than nonstructural virus proteins during HAV replication in cell cultures, and this may be relevant to virus replication and antibody response in vivo (20).

Furthermore, antibody specificities for these individual capsid proteins were investigated. Four antibodies (anti-VP1, anti-
VP2, anti-VP3, and anti-VP4) from guinea pig sera were used to confirm the specific peptide sequences of capsid proteins from the purified HAV antigens. The specificities of these peptides can be distinguished by comparing Fig. 3B and C. The migration characteristics of VP1, VP0 (VP2 plus VP4), and VP3 are different. In contrast, VP2, VP4, and VP0 of HAV seem to have the same mobility on the gel. As shown in Fig. 3C, all of the antisera reacted well in the immunoblot with the respective antigens. This indicates that the prepared HAV particles contained antigenic epitopes for anti-VP1 (lane 1; 33.2 kDa), anti-VP2 (lane 2; 29.1 kDa; in fact, anti-VP0), anti-VP3 (lane 3; 27.2 kDa), and anti-VP4 (lane 4; 29.1 kDa; in fact, anti-VP0) antibodies. VP0 of our HAV strain was shown to be uncleaved into VP2 and VP4. On the basis of this observation, the reactivity of sera to VP0 could be thought to be reactivity to both VP2 and VP4.

Subsequently, the reactivity of human sera to VP1, VP0, and VP3 was identifiable through this assay. This purified HAV was thus used as a substrate for binding of the antibodies (from patients) with the separated capsids on the immunoblot. By using this method, we classified the reactions of the anti-HAV total antibodies and the anti-HAV IgM antibodies, both of which included antibodies to structural proteins VP1 (anti-HAVVP1), VP0 (anti-HAVVP2 + VP4), and VP3 (anti-HAVVP3).

Serum samples from one HAV-infected patient were chosen to demonstrate the efficacy of this method. The sera were incubated with the separated antigen immobilized on the blot and treated as described in Materials and Methods. As illustrated in Fig. 4, the reactivity of anti-HAV IgG (A) and IgM (B) antibodies with VP1 and VP3 can easily be distinguished on the nitrocellulose blot. In our data, no antibodies against the nonstructural antigen were detected. A substantial proportion of sera that were positive for total anti-HAV antibodies were positive only for anticapsid antibodies.

**Acute-phase and long-term reactivities of anti-HAV IgG and IgM antibodies to capsid proteins in HAV-infected sera.** In testing the reactivity of anti-HAV IgG to viral capsids during the acute phase of infection, we found that 22 sera reacted well with all three capsid proteins (Fig. 5). The other sera reacted with either VP1, VP0, VP3, or combinations thereof. In this experiment, no correlation between the anti-HAV total antibody titer and reactivity to the structural proteins was observed. We observed, in all of our samples, that IgM clearly reacted with VP1. Only some sera reacted with VP1. Five serum samples reacted with VP1 and VP0, but antibodies against VP1 and VP3 were present in only two samples. In addition, 10 serum samples reacted to all of the three capsid proteins (Fig. 5). In summary, the appearance of anti-HAVVP1, anti-HAVVP0, and anti-HAVVP3 antibodies after the onset of icterus is depicted in Fig. 5.

A similar method was used to classify the anti-HAV IgG antibodies in 45 long-term serum samples of eight patients. The anti-VP1, anti-VP0, and anti-VP3 activities of the anti-HAV IgG antibodies of these sera were determined and plotted (Fig. 6). Three patients were found to have anti-VP1, anti-VP0, and anti-VP3 activities for periods of 4 to 6 years. By contrast, anti-VP1, anti-VP0, and anti-VP3 were not detected at all after 1 year in two cases. As seen in Fig. 3A, it is worth mentioning that we were able to document an interesting case of a patient whose serum showed that anti-VP3 activity persisted, whereas anti-VP1 and anti-VP0 activities remained undetectable. Data on long-term anti-HAV IgG reactivity to HAV capsid proteins are summarized in Fig. 6.

**DISCUSSION**

In this study, antibody responses to individual HAV capsid proteins (VP1, VP0 [VP2 and VP4], and VP3) in human sera were investigated. Serum samples were obtained from 31 patients during the acute infection phase, and 8 of the 31 subjects were available for long-term follow-up (up to 10 years). Our data indicate that antibodies to structural HAV proteins develop after clinical hepatitis. These antibodies were readily detectable with our immunoblotting assay, and they were present for a long time.

Significantly, antibodies to VP1, VP0 (VP2 plus VP4), and VP3 can be detected by our immunoblotting method in many sera. This important fact is somewhat surprising and should be addressed now because most previous work suggests that the dominant antibody response is directed against conformational epitopes that are displayed only by assembled capsid proteins and not by individual capsid proteins. On this point, however, it should be noted that the viral proteins were denatured and separated by SDS-PAGE. Under these conditions, the gel included only a limited number of denatured and linear peptides and contained epitopes that were very likely synthetic peptides of the viral capsid sequences. In theory, such antibodies are not necessarily neutralizing, as seen in experimental infections with hepatitis C virus, for example (5).

Thus, one needs to be more cautious before concluding that long-lasting immunity against any single viral protein confers protection against that virus and that this kind of antibody response is therefore a promising direction for further vaccine development.

Under this experimental restriction, our study of the immune response after natural HAV infection showed that HAV-specific IgM class antibodies favor the recognition of VP1 (94%). Only small amounts of IgM reacted with VP0.
and VP3 (25%). These observations differ from those of other researchers, who have reported that the IgM antibody response is directed primarily against all viral proteins (10).

Antigen quality plays a very important role in determining the results of immunoblot analysis. In our purified HAV antigen, equimolar amounts of all picornavirus proteins are, in theory, produced because the primary translation product is a polyprotein from which individual mature proteins are generated by a series of proteolytic cleavages. Such a series constitutes a factor contributing to the stability of HAV structures, whereas 3D (the gene for RNA-dependent RNA polymerase) is not assembled into these structures and is therefore susceptible to turnover. Antibodies against the nonstructural antigen are thus shorter-lived than capsid antibodies. This might be the reason for the absence of anti-nonstructural proteins in all of the serum samples from patients (19).

Interestingly, VP0 fragments should cleave into VP2 and VP4, as expected by analogy with other picornaviruses. The presence of only VP0 suggests that the viral antigen is composed largely, if not entirely, of empty capsids. However, our purified virus must have contained mature virus, according to its CsCl density (1.23 to 1.33 g/cm³). Only VP0 fragments, and not separated VP2 and VP4 capsid proteins, were detectable, suggesting that VP0 of the HFS/GBM strain in our preparation might not have been cleaved into VP2 and VP4 after maturation. This result is different from that of a previous report, which showed the presence of VP0 and VP2 in mature virus particles cultivated by fetal rhesus monkey kidney cells (20). Further investigation is required to resolve this puzzling result.

Although the immunodominance of VP1 (98%) in the HAV IgG response of acute-phase sera has been illustrated, anti-VP0 (81%) and anti-VP3 (88%) activities were also detected in most of these sera. In conducting a long-term follow-up of antibodies to the individual HAV capsids in sera, we analyzed sera taken 4 to 10 years after the onset of HAV disease. Most of these samples reacted with viral capsid protein VP1, VP0, or VP3. There was only a single case in which serum reacted not only strongly but also exclusively with VP3 after 10 years of follow-up (Fig. 4), which is evidence that the immune response to HAV infection varies significantly from individual to individual. Another explanation could be that different variants of viral strains elicit different antibody responses.

![FIG. 3. (A) Purification of HAV by CsCl gradient analysis. Fractions separated by a CsCl gradient are shown from the left (top of the gradient) to the right (bottom of the gradient). Twenty-four fractions were collected, and each fraction was monitored for density and antigen content by a direct RIA. (B) SDS-PAGE of HAV preparation. Right lane, most highly concentrated, purified HAV (RIA titer, 1:50,000); lane M, protein molecular size standard (Pharmacia, Uppsala, Sweden). Viral proteins VP1, VP0, and VP3 were the most abundant proteins in the preparation. Entire proteins were stained with ponceau red after blotting. (C) Immunoblot of HAV capsids detected by polyclonal antibodies. Lanes: 1, anti-VP1; 2, anti-VP2; 3, anti-VP3; 4, anti-VP4 guinea pig sera. Purified HAV was separated by PAGE, immobilized on a membrane, and stained with anti-VP1, anti-VP2, anti-VP3, and anti-VP4 sera as described in Materials and Methods. HAV VP1 (31.2 kDa), VP2 (29.1 kDa; in fact, in uncleaved VP0), VP3 (28.2 kDa), and VP4 (29.1 kDa; in fact in uncleaved VP0) viral capsid polypeptides were well recognized on the blot. Proteins with standard molecular weights from similar gels were transferred to nitrocellulose membranes and stained with ponceau red.

![FIG. 4. Immunoblot of HAV capsids detected in serum samples collected from a patient showing anti-HAV IgG (A) and IgM (B) immune responses to VP1 and VP3. Reactivity of anti-HAV IgG (h) and IgM (F) antibodies to capsid proteins in sera from patients in acute phase of HAV infection. The appearance of anti-HAV VP1, anti-HAV VP0, and anti-HAV VP3 antibodies in 31 acute-phase serum samples collected on various occasions after the onset of icterus from patients with hepatitis A was determined and plotted.

![FIG. 5. Reactivity of anti-HAV IgG (C) and IgM (●) antibodies to capsid proteins in sera from patients in acute phase of HAV infection. The appearance of anti-HAV VP1, anti-HAV VP0, and anti-HAV VP3 antibodies in 31 acute-phase serum samples collected on various occasions after the onset of icterus from patients with hepatitis A was determined and plotted.]
Unfortunately, anti-VP1, anti-VP0, and anti-VP3 neutralizing antibodies were impossible to determine in this study. Nevertheless, according to some researchers (9), the profile of an anti-HAV neutralizing antibody correlates closely with that of an anti-HAV (predominantly anti-HAV IgG) binding antibody. This indicates the possible existence of neutralizing anti-VP1, anti-VP0, and anti-VP3 antibodies in the sera of HAV-infected patients. However, our data demonstrate that detection of antibodies to individual capsid proteins by immunoblotting does not correlate with the titre of anti-HAV antibodies as detected by immunoassays using native viral antigen. For example, in Fig. 4, lanes 3 and 14 represent results obtained with serum containing 31,000 and 38,000 mIU of anti-HAV antibodies per ml, respectively. Only lane 3 shows the content of serum samples correlating closely with neutralizing antibody activity, which suggests that antibodies detected by the immunoblotting method do not correlate with protection and are likely to represent only minor species of antibodies that play a questionable role in protection against the virus.

In general, comparisons of immune responses to a native viral antigen and a denatured or synthetic peptide derived from it may have implications for the development of an HAV vaccine. The results described here present several interesting features. (i) A denatured capsid peptide represents only a fraction of the native antigen sequence since it necessarily contains only a limited repertoire of the B-cell epitopes. However, in our study, this kind of antibody population was shown by our immunoblotting method to persist for over 10 years in one patient, although some patients are not responsive to these peptides. However, as mentioned above, nonresponse to the denatured capsid antigen might afford protection at B- and T-cell levels that are not detectable by our immunoblotting method, which is directed only to nonnative viral epitopes. In turn, this result suggests that a vaccine formulation that includes both native antigens and the linear peptides derived from these proteins might be effective in eliciting an immune response, as in the case of hepatitis B vaccine (3, 17). (ii) At least in the present instance, our alum-absorbed, killed-HAV vaccine (6) was able to more efficiently recruit a B-cell response only to the capsid protein in humans (7, 22).

In this context, it is somewhat intriguing that responsiveness to linear capsid proteins might appear to be due to the presence of T cells capable of recognizing appropriately presented HAV-derived epitopes. Does the responsiveness to the capsid peptides by the immunoblotting technique provide additional information and tools for fine analysis of the parameters that influence either immune response or, more importantly, a lack of it? Incidentally, whether or not the individual capsid peptides of HAV are able to prime T cells relative to immunization with a native antigen remains to be determined. The delineation of these regulatory influences should aid in the design and development of more effective vaccines.

In this study, the question of whether or not the capsid peptides derived from native antigen are important in eliciting an immune response in a broader cross-section of the target population could not be answered. Nevertheless, on the basis of our findings, we suggest that the immune response of a host after HAV infection was composed mostly of antibodies against all three viral capsid peptides. These antibodies are present over an extended period in the sera of hepatitis A patients, and it is possible that, at least in most patients, these antibodies are of importance in long-lasting immunity. In addition, this technique might provide an additional tool for definitive analysis of the molecular parameters that influence immune response after vaccination.

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