JOURNAL OF VIROLOGY, Feb. 1993, p. 1080–1085
0022-538X/93/021080-06$02.00/0

Vol. 67, No. 2

# Antigenic and Immunogenic Properties of Recombinant Hepatitis A Virus 14S and 70S Subviral Particles

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Received 27 July 1992/Accepted 22 October 1992

**Hepatitis A virus (HAV) has an immunodominant neutralization antigenic site. By using a panel of monoclonal antibodies targeted against the HAV neutralization antigenic site, it was shown that three epitopes within this site are present on 14S subunits (pentamers of the structural unit). In contrast, two other epitopes within this site are formed upon assembly of 14S subunits into capsids. Thus, the epitopes recognized by these two monoclonal antibodies are formed either by a conformational change in the antigenic site or by the juxtaposition of epitope fragments present on different 14S subunits during assembly of 14S into 70S particles. Both 14S and 70S particles elicited HAV-neutralizing antibodies in mice; thus, these particles may be useful for HAV vaccine development.**

Hepatitis A virus (HAV) is a medically important member of the picornavirus family. Although the specific study of HAV assembly and antigenic structure has been hampered by its slow growth and relatively low yield in tissue culture (23), HAV morphogenesis is thought to be similar to that of poliovirus, the prototype picornavirus. Poliovirus capsids are assembled from 12 subunits called pentamers which sediment at 14S. These subunits contain five copies of a "protomer" (5S) which consists of one molecule of each of the capsid proteins 1AB (VP0), 1C (VP3), and 1D (VP1) (22). HAV pentamers have a sedimentation coefficient of 14S (1), and RNA-containing virions sediment at approximately 150S (23). In addition, HAV 70S (empty capsid) and 135S RNA-containing particles have been described (1, 21, 29).

Four neutralization antigenic sites have been recognized on poliovirus type 1 (14). Three of the four poliovirus type 1 antigenic sites are present on 14S subviral particles, while one site is formed when 14S subunits assemble into empty capsids (18). Less is known about HAV antigenic structure; however, there is an immunodominant neutralization antigenic site on HAV virions and empty capsids which is conformation dependent (16, 28, 29). To circumvent the slow replication cycle and low levels of protein synthesis found in HAV-infected cells, we cloned the HAV open reading frame into a vaccinia virus transfer vector and selected recombinant viruses which expressed the HAV polyprotein (rV-ORF) (31). The polyprotein underwent proteolytic processing into HAV capsid proteins, which assembled into 14S and 70S antigenic material recognized by polyclonal human convalescent serum in solid-phase radioimmunoassays (RIA) (31). The 70S antigenic material cosedimented with HAV 70S particles (31). Since rV-ORF did not contain the 5′ nontranslated region of the HAV genome, RNA encapsidation did not occur (31).

We utilized recombinant 14S and 70S HAV antigenic particles to further characterize HAV antigenic structure. By using a panel of neutralizing monoclonal antibodies, HAV neutralization epitopes were localized on 14S and 70S particles. Recombinant vaccinia viruses expressing the HAV polyprotein under the control of the vaccinia virus p7.5 early-late promoter were used to infect confluent human $TK^-$ cell layers as previously described (31). Walter Reed strain vaccinia virus-infected human $TK^-$ cell lysates served as the negative (wild-type) control. Cell lysates ($0.5 \times 10^7$ to $1 \times 10^7$ infected cells) were layered onto 7.5 to 45% (wt/vol) sucrose gradients in 20 mM Tris–10 mM NaCl–50 mM $MgCl_2$, pH 6.7. Gradients were centrifuged for 165 min (205,000 × *g*, 4°C) in an SW41 rotor (Beckman Instruments), and 500-μl fractions were collected from the bottom of the gradient.

To identify neutralization epitopes present on the recombinant 14S and 70S particles, a previously described RIA was employed (3, 9, 31). This RIA utilized different antibodies to capture HAV antigen to the solid phase. Previously characterized HAV-immune and -nonimmune human serum (27, 31), HAV-neutralizing monoclonal antibody (murine and human) (5, 15, 16, 28), and a murine monoclonal antibody directed against myeloperoxidase served as independent capture antibodies. Gradient fractions were applied to wells coated with antibody, and after extensive washing, $^{125}$I-labeled polyclonal anti-HAV immunoglobulin G (IgG) was added for 4 h at 4°C (200,000 cpm per well) (9). The wells were washed, and the amount of $^{125}$I IgG bound to the well was determined (9). Sedimentation markers consisted of HAV 156S and 70S as previously described (31). In addition, human IgM (19S) and IgG (7S) antibody were layered onto individual sucrose gradients (10 μg per gradient) and centrifuged simultaneously with the vaccinia virus-infected cell lysates. Fractions collected from the bottom of the tube were electrophoresed on sodium dodecyl sulfate–10% polyacrylamide gels, and immunoglobulin-containing fractions were identified by Coomassie blue staining.

Figure 1 demonstrates that HAV-immune human serum (polyclonal) (27), HAV-neutralizing murine monoclonal antibodies B5B3, K2-4F2, K3-4C8, K3-2F2 (28), and HAV-neutralizing human monoclonal antibody 3.2.4 (5) detected comparable amounts of HAV and recombinant 70S HAV

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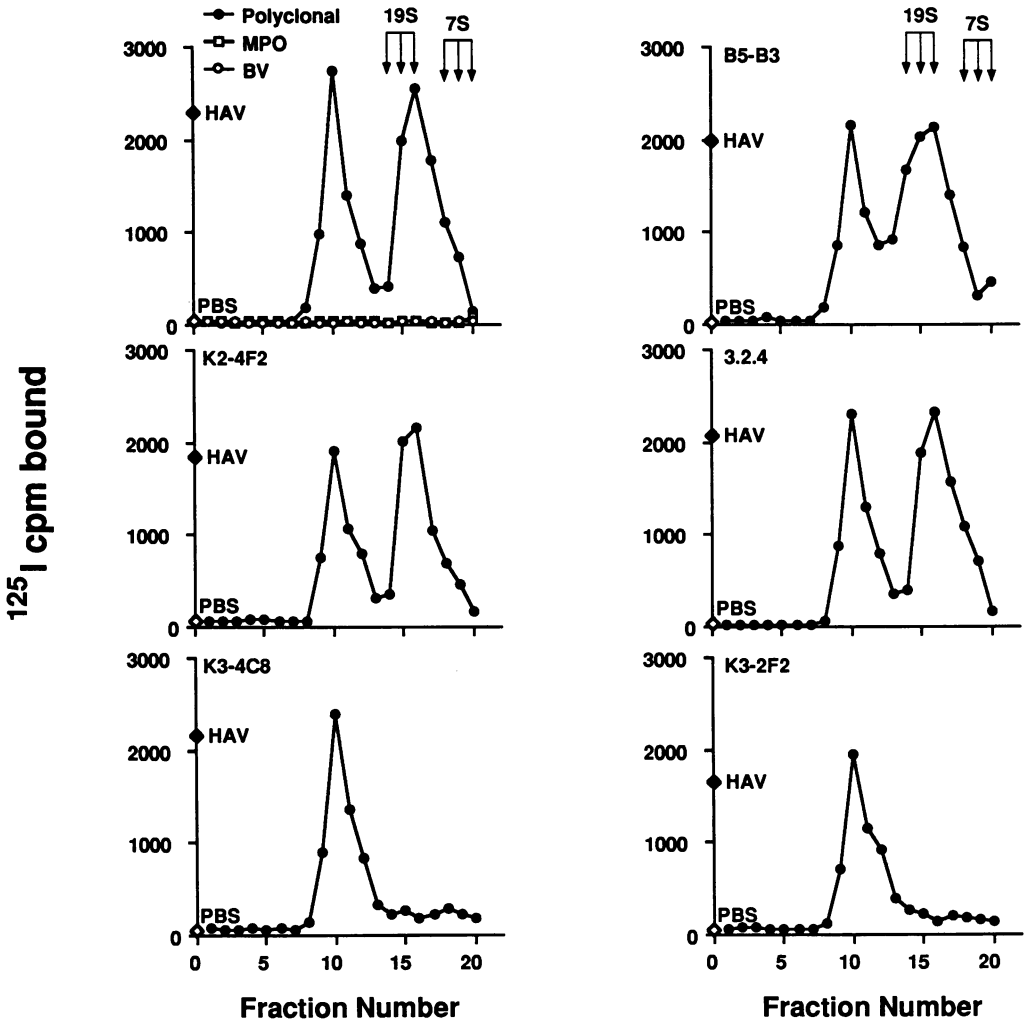


FIG. 1. Cells infected with recombinant vaccinia virus expressing the hepatitis A virus open reading frame (rV-ORF) were lysed and fractionated on a single 7.5 to 45% sucrose gradient. Gradient fractions were collected from the bottom of the tube and analyzed by RIA employing polyclonal and monoclonal antibodies. The top left panel demonstrates results obtained when HAV-immune human serum served as the capture antibody (polyclonal), and when HAV-nonimmune human serum or a monoclonal antibody directed against myeloperoxidase (BV and MPO, respectively) were used. The top right and middle panels demonstrate results obtained by using murine monoclonal ascitic fluids (B5B3 or K2-4F2) or human monoclonal cell supernatant fluid (3.2.4) as the capture antibody. The lower panels demonstrate results obtained by using K3-4C8 and K3-2F2 monoclonal antibodies. IgG (7S) and IgM (19S) markers fractionated simultaneously sedimented in the fractions indicated. Standard HAV antigen (◆) and PBS control (◇) counts per minute bound are indicated. Similar results have been obtained from three independent rV-ORF-infected cell lysates.

antigen, indicating that all of these neutralization epitopes were present on HAV empty capsids. 14S HAV antigen was detected by HAV-immune human serum and K2-4F2, B5B3, and 3.2.4 monoclonal antibodies. However, the two closely related murine HAV monoclonal antibodies, K3-2F2 and K3-4C8 (13, 28), did not detect antigen from the 14S fractions, suggesting that the formation of these epitopes required assembly of 14S into 70S empty capsids. HAV-nonimmune human sera and a monoclonal antibody directed against myeloperoxidase did not detect HAV-antigenic material.

High-performance size-exclusion liquid chromatography (HPSELC) has been used to separate poliovirus 70S empty capsids from 14S pentamers (7). When rV-ORF infected TK$^-$ cell lysates ($10^6$ cells) were fractionated by HPSELC with two Ultrahydrogel 1000 columns (Waters, Milford, Mass.) in series as described by Foriers et al. (7), two HAV-antigenic peaks were eluted at 70.1 and 78 min. The antigen eluted at 78 min sedimented at 14S in sucrose gradients and was not detected by K3-4C8 antibody, whereas the antigen eluted at 70.1 min had a sedimentation of 70S and was recognized by all three monoclonal antibodies (data not shown).

Concentration of purified poliovirus 14S particles results in self-assembly into 70S empty capsids (19, 20). To determine whether HAV-antigenic material sedimenting at 14S would assemble into 70S particles, 14S-containing fractions from six gradients were pooled, concentrated 10-fold in a Centriprep 30 concentrator (Amicon, Danvers, Mass.), incubated 1 h at 37°C, and layered onto a sucrose gradient (Fig. 2). Gradient fractions were evaluated by RIA as described above. Following concentration of the 14S pool,

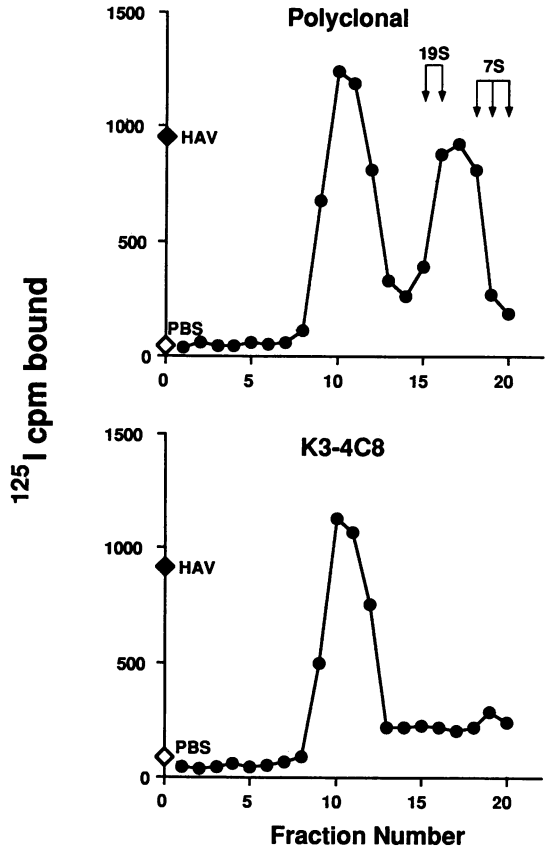


FIG. 2. 14S HAV antigen from six sucrose gradients was pooled, concentrated 10-fold, incubated for 1 h at 37°C, and centrifuged over another 7.5 to 45% sucrose gradient. Gradient fractions were analyzed by either polyclonal radioimmunoassay (RIA) (top panel) or K3-4C8 monoclonal RIA (bottom panel). The binding of HAV (positive control) (◆) and background counts per minute (negative control) (◇) were comparable when these antibodies were used. Sedimentation markers are indicated as in Fig. 1.

HAV-immune human (polyclonal) serum detected HAV antigen in both 14S and 70S fractions. K3-4C8 antibody did not detect the original, unconcentrated 14S antigen or the antigen which sedimented at 14S following 10-fold concentration. However, K3-4C8 detected HAV antigen in the 70S fractions of this gradient, indicating that the epitope recognized by K3-4C8 was generated during concentration of 14S antigenic material.

To verify that the 70S material represented HAV-specific subvirus-like particles, immune electron microscopy was carried out. K2-4F2 monoclonal antibody was incubated with 70S antigenic material from the gradient shown in Fig. 2 as previously described (31). Immune complexes were pelleted, and the pellet was resuspended in 50 μl of phosphate-buffered saline (PBS), applied to carbon-coated Formvar grids, and negatively stained with 2% phosphotungstic acid. Grids were evaluated by using a Hitachi 7000 electron microscope (University of Iowa Electron Microscope Facility). Particles (27 nm) coated with antibody were detected in these 70S fractions, consistent with empty capsids (Fig. 3).

To determine the degree of concentration required for 14S antigen to generate the K3-4C8 epitope, 14S and 70S fractions were separately pooled following sucrose gradient

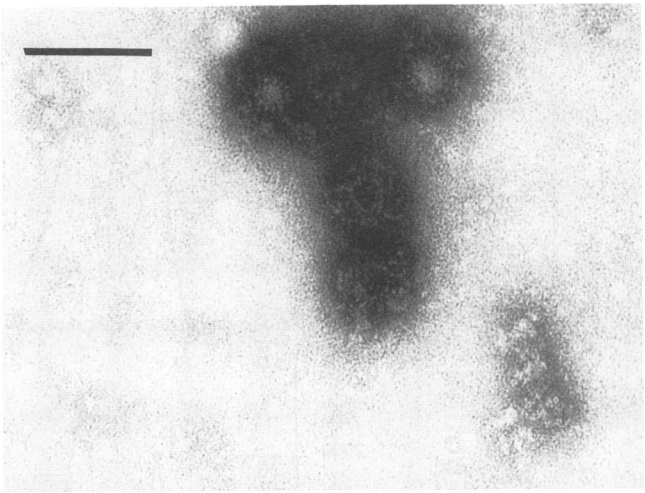

FIG. 3. 70S HAV-antigenic material from fractions 9, 10, and 11 (Fig. 2) was pooled and incubated with K2-4F2 monoclonal antibody, and immune complexes were pelleted as previously described (31). Pellets were resuspended, negatively stained with phosphotungstic acid, and evaluated by electron microscopy. Bar, 100 nm.

centrifugation. An aliquot of each pool was removed for subsequent RIA testing. The two pools were independently concentrated in twofold increments, with an equal volume removed after each concentration step. These samples were incubated at 37°C for 1 h and assayed for HAV antigen by RIA using either polyclonal or K3-4C8 monoclonal antibody as the capture antibody (Fig. 4). In the unconcentrated and twofold-concentrated 14S samples, HAV antigen was detected by polyclonal HAV antibody but not K3-4C8 monoclonal antibody. Following fourfold or greater concentration of 14S antigen, both polyclonal and K3-4C8 monoclonal antibody detected the antigen, indicating that the epitope recognized by K3-4C8 antibody was generated in the concentrated sample. Polyclonal antibody recognized both 14S and 70S particles, regardless of the degree of concentration. On the basis of the sucrose gradient centrifugation data described in the legends to Fig. 1 and 2, the generation of the K3-4C8 epitope suggests that assembly of 14S particles into 70S particles occurred. Therefore, this monoclonal antibody-based RIA method is a simple and rapid way to measure assembly of HAV 14S particles and to distinguish 70S from 14S antigen.

Replication of HAV in cell culture generates a variety of virus particles (6, 21), and a significant portion of recombinant HAV antigen present in rV-ORF infected cells is in the form of 14S particles (31). Therefore, we wished to determine whether either the recombinant 14S or 70S antigenic particles were immunogenic. Groups of four mice were immunized with either recombinant 14S or recombinant 70S HAV antigen purified by sucrose gradient centrifugation. HAV strain HM-175 grown in BS-C-1 cells and partially purified by cesium chloride gradient purification (8, 25, 26) served as the positive control immunogen. This HAV preparation contained a mixture of 156S (infectious) and 70S HAV antigen (data not shown). HAV antigen content in each preparation (14S, 70S, HAV) was quantitated by RIA and standardized by comparison with $1.2 \times 10^5$ infectious units of HAV (8, 26), an antigen dose which falls in the linear range of $^{125}$I counts per minute bound in the RIA used. This antigen concentration was comparable to 25 enzyme-linked

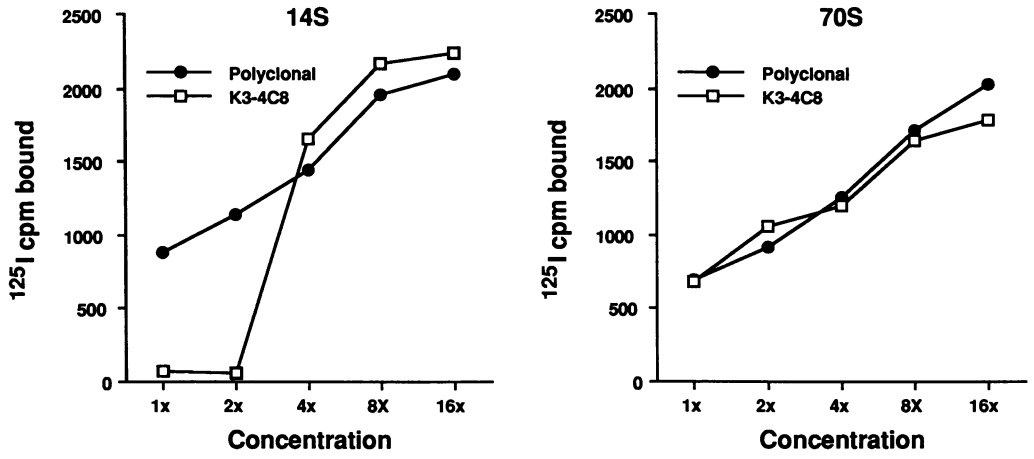


FIG. 4. 14S (left panel) and 70S (right panel) particles obtained following sucrose gradient sedimentation were independently concentrated in serial twofold increments (Centriprep concentrators; Amicon). Aliquots were removed after each step, incubated for 1 h at 37°C, and assayed by HAV-immune human serum (polyclonal) antibody-based RIA or K3-4C8 monoclonal antibody-based RIA. Results are expressed as the mean $^{125}I$ counts per minute bound in duplicate wells. Similar results were obtained in three separate experiments.

immunosorbent assay (ELISA) units of HAV antigen, defined for a commercially available inactivated HAV vaccine preparation (kindly provided by Eric D'Hondt, SmithKline Beecham Laboratories, Rixensart, Belgium) (30). Following adsorption to alum (50 μg per mouse), twofold concentrations of antigen were resuspended in PBS and administered to mice intraperitoneally (four mice per study group). Alum in PBS was administered as the negative control. Animals were boosted 3 and 6 weeks after the initial immunization for 70S and HAV immunizations and boosted once 6 weeks after the initial immunization for 14S particles. Table 1 demonstrates the mean antibody titer in mIU (26) for each group, as determined by commercial ELISA (HAVAB; Abbott Laboratories, North Chicago, Ill.). Positive sera neutralized HAV infectivity by radioimmunofocus inhibition test at a 1:100 dilution (26). The immunopotency of recombinant 14S particles was 20- to 30-fold less than that of the recombinant 70S particles and HAV; however, the different immunization schedule obviates a direct comparison. Nevertheless, mice within each immunization group seroconverted, while none of four control mice receiving 50 μg of alum in PBS seroconverted.

These data contribute to the growing understanding of HAV-antigenic structure. HAV-antigenic sites are strictly conserved among human HAV isolates collected from diverse geographic locations (10). HAV-neutralizing antibodies appear to be closely related, as they extensively compete for binding to the surface of HAV (28). Some monoclonal antibodies, including the K2-4F2 antibody used in this study, compete with all monoclonal antibodies described to date (16). B5B3 antibody does not compete with either K3-2F2 or K3-4C8 antibodies and in fact enhances the binding of these antibodies to the virus, indicating that these antibodies bind to distinct epitopes within this antigenic site (28). The combination of K3-4C8 and B5B3 antibodies competed almost completely with polyclonal human HAV convalescent antibody, suggesting that the antigenic site recognized by these antibodies is immunodominant in mice and in humans (28). Neutralization escape mutants selected for resistance to K2-4F2 and B5B3 antibodies demonstrated cross-resistance of various degrees to both K3-4C8 and B5B3 monoclonal antibodies, further suggesting that the epitopes recognized by these antibodies comprise an immunodominant neutralization antigenic site (28).

TABLE 1. Immunogenicity of recombinant 14S and 70S particles in mice

| Immunogen | Immunogenicity[a] at HAV antigen concn[b] | | | |
|---|---|---|---|---|
| | 25 | 50 | 100 | 200 |
| 14S | 1,050 (2/4) | 3,000 (2/4) | 6,600 (4/4) | NT[c] |
| 70S | 39,000 (3/4) | NT | NT | 62,400 (4/4) |
| HAV | NT | 57,600 (3/4) | NT | 258,000 (3/3) |

[a] Results are expressed as mIU of HAV antibody (no. of seroconverters/no. immunized as determined by ELISA [HAVAB; Abbott Laboratories]).
[b] Antigen concentration is expressed as HAV ELISA units; 25 ELISA units corresponds to $1.2 \times 10^5$ infectious HAV units in BS-C-1 cells (see text).
[c] NT, not tested.

Mutation of the Asp-70 or Gly-74 residue of capsid protein VP3, or Ser-102, Ser-114, Val-171, or Ala-176 of capsid protein VP1, results in neutralization escape phenotype (4, 15, 16). Mutant viruses demonstrate partial or complete resistance to neutralization by monoclonal antibodies directed against epitopes within the immunodominant neutralization site (4, 15, 16). In addition, a second, potentially independent site involving Lys-221 of VP1 was recently identified (16). No HAV neutralization escape mutants have been selected by using K3-4C8 or K3-2F2 monoclonal antibodies to date (16, 28). However, VP3 Asp-70 mutations resulted in partial resistance to neutralization by both of these antibodies (16, 28); thus K3-4C8 and K3-2F2 either bind to part of a large antigenic site involving several epitopes which include VP3 Asp-70, or they bind to a separate site on the virion that undergoes a conformational change when VP3 Asp-70 is altered. The fact that K3-4C8 and K3-2F2 monoclonal antibodies recognized 70S and not 14S particles indicates that the HAV-immunodominant neutralization antigenic site undergoes a structural change during assembly of 14S particles leading to the formation of these neutralization epitopes.

Amino acid residues identified to be involved in neutralization antigenic sites on HAV capsid proteins VP3 and VP1 align with neutralization antigenic sites present within the

βB and βC loops of poliovirus type 1 capsid proteins (11, 14, 15). Thus, there appears to be structural conservation of antigenic sites on these viruses, in spite of the lack of amino acid homology (2, 10). Poliovirus type 1 contains a neutralization antigenic site (3B) which contains VP3 amino acid 76 and VP2 amino acids 72, 74, 243, and 246 (14, 17). The formation of this site requires assembly of 14S subunits into empty capsids (18), with residues from one region of the pentamer brought into close proximity with a different region of an adjacent pentamer during assembly, creating the neutralization epitope. Our results are compatible with the hypothesis that the HAV epitopes recognized by K3-4C8 and K3-2F2 antibodies are formed in an analogous manner. Comparing the nucleotide sequence alignments and X-ray crystallographic data from other picornaviruses, Luo et al. predicted a map of the HAV surface (12). Amino acid residues surrounding the VP3 Asp-70 site (VP3 amino acids 68, 71, and 73) were predicted to reside on the surface near the threefold axis of the virion (12), similar to the location of the poliovirus 3B site (14, 17). Further characterization of this neutralization antigenic site on HAV will require either the selection and characterization of K3-4C8 or K3-2F2 resistant escape mutants or determination of the three-dimensional structure of HAV by X-ray crystallography.

Previous studies have not reported the immunogenicity of HAV 14S or 70S particles. Given the antigenic composition of 70S particles, it was not surprising that they elicited neutralizing antibodies in mice. This is relevant to current inactivated HAV vaccines, since more than 60% of antigen in HAV-infected cell cultures is in the form of empty capsids (29), (reviewed in reference 24). Although recombinant 14S particles do not contain all identified neutralization epitopes, they do contain several epitopes recognized by neutralizing monoclonal antibodies (Fig. 1). Immunization of mice with 14S particles elicited levels of neutralizing antibody which were three orders of magnitude greater than the level of neutralizing antibody found in humans passively immunized with pooled serum immunoglobulin (26). Since immunoglobulin administration decreases HAV infection and disease by approximately 90% (reviewed in reference 32), it appears that both the recombinant 14S and 70S particles have promise as candidate vaccines.

We thank Shankar Iyer for assistance with high-performance liquid chromatography experiments; Barbara Meyer and Eric Johnson for technical assistance; William Nauseef (anti-myeloperoxidase), Richard Tedder (B5B3), Stephen Day, and Stanley Lemon (3.2.4) for monoclonal antibodies; and Naomi Erickson for manuscript preparation.

This work was supported in part by a Merit Review grant from the Veterans Administration (J.T.S.), by The University of Iowa Graduate College for work done in the Electron Microscope Facility (J.T.S.), and by a grant from American Biogenetic Sciences, Inc. (J.T.S.). P.L.W. (associate investigator) and J.T.S. (research associate) are recipients of Veterans Administration Career Development Awards.

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