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Identification of an immunodominant antigenic site involving the capsid protein VP3 of hepatitis A virus

(neutralization/epitope mapping/monoclonal antibodies)

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ABSTRACT **Hepatitis A virus, an hepatotropic picornavirus, is a common cause of acute hepatitis in man for which there is no available vaccine. Competitive binding studies carried out in solid phase suggest that neutralizing monoclonal antibodies to hepatitis A virus recognize a limited number of epitopes on the capsid surface, although the polypeptide locations of these epitopes are not well defined. Neutralization-escape mutants, selected for resistance to monoclonal antibodies, demonstrate broad cross-resistance to other monoclonal antibodies. Sequencing of virion RNA from several of these mutants demonstrated that replacement of aspartic acid residue 70 of capsid protein VP3 (residue 3070) with histidine or alanine confers resistance to neutralization by monoclonal antibody K2-4F2 and prevents binding of this antibody and other antibodies with similar solid-phase competition profiles. These results indicate that residue 3070 contributes to an immunodominant antigenic site. Mutation at residue 102 of VP1 (residue 1102) confers partial resistance against antibody B5-B3 and several other antibodies but does not prevent antibody attachment. Both VP3 and VP1 sites align closely in the linear peptide sequences with sites of neutralization-escape mutations in poliovirus and human rhinovirus, suggesting conservation of structure among these diverse picornaviruses. However, because partial neutralization resistance to several monoclonal antibodies (2D2, 3E1, and B5-B3) was associated with mutation at either residue 3070 or residue 1102, these sites appear more closely related functionally in hepatitis A virus than in these other picornaviruses.**

Hepatitis A virus (HAV), a human picornavirus with tropism for the liver, is responsible for both endemic and epidemic hepatitis worldwide (1). Despite efforts to develop both inactivated and attenuated vaccines, a practical HAV vaccine has yet to be produced for use in human populations. Although the virus may be propagated *in vitro* in a variety of primate cell lines (2–4), the yields of virus are generally low and no clear cut markers of viral attenuation have yet been identified. Similarly, attempts to express viral antigen from recombinant cDNA have not resulted in immunogenic proteins capable of eliciting neutralizing antibody (5, 6), reflecting in part the highly conformational nature of the relevant antigenic sites (7). Despite these difficulties, there is evidence that the antigenic activity of the virus is comprised of relatively few epitopes binding neutralizing antibody (8). We have attempted to identify the molecular structures that contribute to these immunogenic neutralization epitopes. As the corresponding epitopes are well characterized for two distantly related human picornaviruses with known three-dimensional structures, poliovirus type 1 (PV1) (9) and human rhinovirus type 14 (HRV14) (10), identification of HAV neutralization epitopes is also of interest from a comparative viewpoint.

Virus mutants resistant to monoclonal antibody-mediated neutralization have proven helpful in identifying immunogenic sites on these other picornaviruses (9–11), but the application of this approach to characterization of HAV antigenic sites has been hampered by the slow and inefficient replication of HAV *in vitro*. Most HAV isolates are noncytopathic, are highly cell-associated, and demonstrate a substantial nonneutralizable fraction *in vitro* (4, 12). To overcome these problems, a radioimmunofocus assay for HAV, based on the immune autoradiographic detection of virus replication foci developing beneath agarose overlays, has been used to clonally isolate HAV-neutralization-escape mutants resistant to a panel of neutralizing monoclonal antibodies (8, 13). In this report, we describe the further characterization of these monoclonal antibodies and their related neutralization-escape mutants.

METHODS

Monoclonal Antibodies to HAV. Monoclonal antibodies to HAV were obtained from five laboratories (14, 15) (see Table 1). Each was tested for its ability to neutralize the HM175 strain of HAV in a radioimmunofocus-inhibition assay (16) and to compete with other radioiodinated monoclonal antibodies (K3-4C8 or B5-B3) for binding to virus as described (8).

Selection of HAV-Neutralization-Escape Mutants. Spontaneous neutralization-escape mutants were selected from cell-culture-adapted HM175 virus stock by repeated cycles of neutralization that were followed by amplification in the presence of neutralizing monoclonal antibodies, as described (8). Mutant virus was clonally isolated from radioimmunofocus assay agarose overlays (17) and assessed for neutralization resistance in a logarithmic reduction radioimmunofocus-inhibition assay (8, 16).

Amplification and Purification of HAV Mutants. Virus was propagated in 850-cm^2 roller bottle cultures of BS-C-1 cells in the absence of antibody and purified from cell lysates by

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Abbreviations: HAV, hepatitis A virus; PV1, poliovirus type 1; PV3, poliovirus type 3; HRV14, human rhinovirus type 14.

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Table 1. Neutralizing monoclonal antibodies to HAV

Antibody	Virus strain	Isotype	Neut. index	Source
Group A				
K2-4F2	HM790	IgG2a	1.43 ± 0.31	A. MacGregor*
K3-4C8		IgG2a	1.22 ± 0.29	
K3-2F2		IgG2a	ND	
Group B				
B5-B3	KMW-1	IgG2a	0.90 ± 0.12	R. Tedder†
Group C				
6A5	CR326	IgG2a	1.62 ± 0.11	J. Hughes‡
1B9		IgG2a	1.22 ± 0.53	E. Emini‡
2D2		IgG2a	2.00 ± 0.20	R. Gerety‡
3E1		IgG2a	1.25 ± 0.12	
Group D				
10.09	CF53	IgG1	2.43 ± 1.14	D. Crevat§
813		IgG3	1.13 ± 0.44	E. Deloince§
Group E				
AG3	S84-1	IgG1	0.69	C. Li¶
AD2		IgG1	0.95	
AE8		IgM	1.81	

Neutralizing index = $\log_{10}$ reduction in titer of HM175 virus (mean ± SEM). Neut., neutralizing; ND, not determined.
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centrifugation through combination sucrose/cesium chloride gradients (18).

Antibody Binding to HAV Mutants. A standardized quantity of gradient-purified virus, measured by quantitative cDNA·RNA hybridization, was applied to the wells of a polyclonal antibody-coated polyvinylchloride microtiter plate (19, 20). After extensive washing, ^{125}I-labeled monoclonal antibody (K2-4F2, K3-4C8, or B5-B3) was added to each well (direct assay). Alternatively, after incubation with excess unlabeled monoclonal antibodies and subsequent washing, radioiodinated goat anti-mouse immunoglobulin (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added to each well (indirect assay). After further washing steps, the quantity of ^{125}I bound to mutant virus was compared with that bound to comparable amounts of the parent HM175 virus.

RNA Sequencing. Gradient-purified virus was treated with $NaDodSO_4$/proteinase K, followed by extraction of RNA with phenol/chloroform (21). HAV RNA was twice ethanol-precipitated and quantified by cDNA·RNA hybridization (20). Sequencing of RNA was accomplished by primer extension in the presence of dideoxynucleotide triphosphates (L.-H.P., R.W.J., and S.M.L., unpublished data).

RESULTS

Competitive Binding of Neutralizing Monoclonal Antibodies to HAV. Thirteen available monoclonal antibodies to HAV neutralized HM175 strain virus when tested individually in a radioimmunofocus-inhibition assay (Table 1). However, previous studies have demonstrated that two of these antibodies, K3-4C8 and B5-B3, are directed against spatially distinct nonoverlapping epitopes on the surface of the virus (8). We examined the ability of each monoclonal antibody to compete with radiolabeled K3-4C8 or B5-B3 for attachment to HAV immobilized on a solid-phase support (Fig. 1). These results suggested that the monoclonal antibodies could be functionally grouped into four general categories: antibodies K3-4C8, K3-2F2, and 813 that strongly compete with K3-4C8 but enhance the binding of B5-B3 to virus; antibodies 6A5, AG3, K2-4F2, and 10.09 that strongly compete with K3-4C8 and weakly compete with B5-B3; antibodies 1B9, 2D2, 3E1, and AD2 that strongly compete with both K3-4C8 and B5-B3; and antibodies AE8 and B5-B3 that compete weakly or not at all with K3-4C8, but strongly compete with B5-B3. Because antibodies in the first group (antibodies K3-4C8, K3-2F2, and 813) do not competitively inhibit attachment of B5-B3 to virus, and B5-B3 does not compete for binding with K3-4C8 (Fig. 1), it may be concluded that nonspecific steric hindrance does not affect the simultaneous attachment of IgG antibodies directed at spatially distinct epitopes on the virus. However, the enhancement of B5-B3 binding after the reaction of virus with antibodies K3-4C8, K3-2F2, or 813 suggests that these antibodies induce conformational changes at the B5-B3 epitope and, therefore, that these epitopes may be closely situated on the virus capsid. This hypothesis is further supported by the inhibition of both K3-4C8 and B5-B3 binding demonstrated by multiple other antibodies (Fig. 1). These data thus suggest that the 13 neutralizing monoclonal antibodies listed in Table 1 are directed against a cluster of closely spaced epitopes. Furthermore, the neutralization results shown in Table 1 and previously reported data (8, 22) suggest that this epitope cluster is highly conserved and immunodominant in man.

Analysis of HAV-Neutralization-Escape Mutants. The selection of spontaneous HAV-neutralization-escape mutants required a process of repeated neutralization and passage in

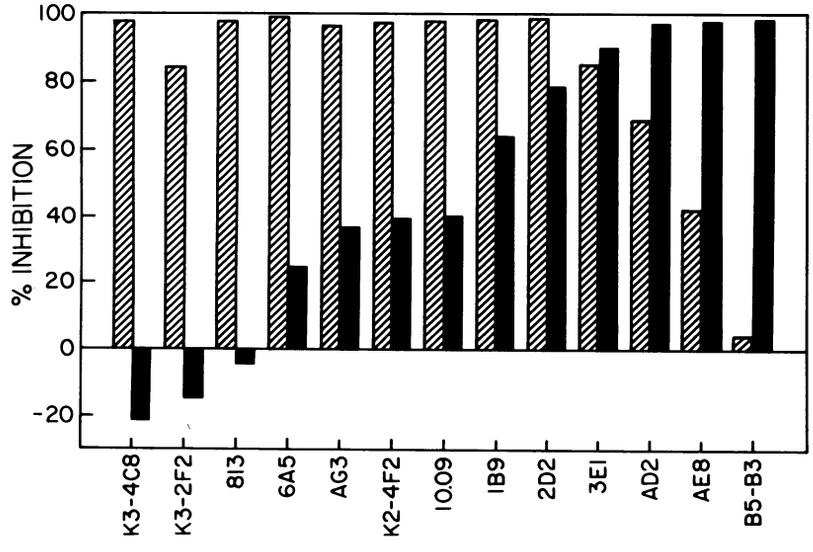

FIG. 1. Competition between monoclonal antibodies for attachment to HAV. Antibody dilutions were added to virus-coated microtiter wells prior to the addition of much smaller quantities of ^{125}I-labeled monoclonal antibody K3-4C8 (hatched bars) or B5-B3 (solid bars). The quantity of ^{125}I bound was compared with that bound in the absence of any competing antibody.

the presence of antibody that was followed by clonal isolation from agarose overlays (8). The neutralization resistance of these mutants was reconfirmed after amplification of virus in the absence of antibody (Table 2) with some variants (e.g., S20), demonstrating partial to complete reversion to neutralization susceptibility. The neutralization resistance of some HAV mutants has been associated with reduced binding of the cognate antibody in immunoaffinity hybridization experiments (8). This was confirmed in solid-phase radioimmunoassays. Radioiodinated antibodies K2-4F2 and K3-4C8 bound to parent HM175 virus but only K3-4C8 bound to the two virus mutants, S18 and S30, that had been selected for resistance to K2-4F2 antibody (data not shown).

To identify capsid protein amino acid mutations associated with neutralization resistance, and by inference involved in antibody attachment sites on the virion surface (9, 10), we partially sequenced RNA extracted from preparations of purified virus. For purposes of comparison, we sequenced the complete capsid-encoding regions (P1 domain) of RNA extracted from mutants S30 and S32 and compared these RNA sequences with sequence we had obtained from cDNA clones of parental virus with a normal neutralization phenotype (21). S30 was selected for resistance to antibody K2-4F2, and the titer of S30 was not decreased when incubated with this antibody, whereas S32 was selected against B5-B3 and demonstrated partial resistance against this antibody (Table 2).

The only mutation identified in the capsid-encoding region of S30 virus was a G → C substitution at base 1677, which predicted a change from aspartic acid to histidine at residue 70 of capsid protein VP3 (residue 3070). [We have adopted the nucleotide numbering system and proposed protein assignments of Cohen *et al.* (23). By convention, we describe the 70th residue of capsid polypeptide VP3 as residue 3070.] Limited RNA sequencing demonstrated that this substitution was also present in four other mutants selected against K2-4F2 (S18, S23, S27, and S28), but absent in mutant S20, which had reverted to neutralization susceptibility during virus amplification (Table 2). Because of the method of mutant selection (8), these viruses may represent sibling variants originally derived from a single neutralization-resistant parent. However, substitution at this same residue was also found in an independently selected, K2-4F2-resistant mutant (43c12), in which an A → C substitution at base 1678 predicted replacement of 3070 with alanine. This variant, which was derived from a rapidly replicating cytopathic HM175 virus substrain (24), has been partially sequenced in the P1 region. It also has an A → G substitution at base 2797 that predicts replacement of asparagine-1197 with serine. Residue 3070 was unchanged in S32 virus (Table 2). The only mutation within the P1 region of this virus was a C → U mutation at base position 2512, predicting a change from serine to leucine at residue 1102.

Table 2. Neutralization-resistant HAV variants

Antibody	Mutant	Neut. index	Control neut. index	Residue 3070	Residue 1102
K2-4F2	S18	−0.12	1.43 ± 0.31	His	Ser
	S20	0.88		Asp	Ser
	S23	0.03		His	Ser
	S27	−0.06		His	Ser
	S28	0.02		His	Ser
	S30	−0.09		His	Ser
	43c12	0.00		Ala	Ser
B5-B3	S32	0.63 ± 0.02	0.90 ± 0.12	Asp	Leu

Neutralization (neut.) index = $\log_{10}$ reduction in virus titer after neutralization with the cognate antibody. Control neutralization index is the neutralization index of parent HM175 virus with same antibody.

Table 3. Resistance of virus mutants to monoclonal antibodies

Antibody	S30 Neut. index	S30 Control neut. index	S32 Neut. index	S32 Control neut. index
K3-4C8	0.25	1.10	0.80	0.93
K3-2F2	−0.14	0.82		
813	−0.09	0.91	0.58	0.73
6A5	−0.03	1.50	1.15	1.73
AG3	1.41	1.13	1.50	1.13
K2-4F2	−0.08	1.33	1.05	1.25
10.09	0.19	1.33	0.60	1.00
1B9	0.31	0.80	0.63	0.89
2D2	0.13	1.80	0.89	2.20
3E1	−0.16	1.10	0.50	1.25
AD2	0.21	0.60		
AE8	1.84	1.81		
B5-B3	0.60	0.92	0.65	1.06
pcAb	0.85	1.21		

Neutralization index and control neutralization index are as defined in Table 2. pcAb, human polyclonal antibody; neut., neutralization.

Immunodominance of the Antigenic Site Containing Residue 3070. We tested S30 virus for resistance to other monoclonal antibodies (Table 3). Neutralization resistance was evident with antibodies with activities similar to the cognate antibody K2-4F2 in competition studies (antibodies 6A5 and 10.09) and also antibody 1B9 (Fig. 1); however, S30 was at least partially resistant to antibodies K3-4C8, K3-2F2, 813, 2D2, 3E1, and B5-B3. The cross-resistance of S30 to other monoclonal antibodies suggests that the epitopes recognized by these antibodies comprise a single functional antigenic domain. Antibodies AE8 and AG3 were notable exceptions in that they retained neutralizing activity against S30 and thus might be directed against an antigenic site distinct from that recognized by the other monoclonal antibodies. We also assessed the neutralization resistance of the VP1 mutant S32 to monoclonal antibodies other than the cognate B5-B3. This mutant was partially resistant to neutralization by antibodies 10.09, 2D2, 3E1, and possibly 6A5, but remained susceptible to K3-4C8, 813, K2-4F2, and 1B9 (Table 3). Complete resistance was not seen to any antibody.

To determine whether resistance of S30 to multiple antibodies was related to loss of antibody binding, we determined the extent of binding of each monoclonal antibody to S30 in an indirect solid-phase radioimmunoassay (Fig. 2). These results indicated that neutralization resistance against antibodies K3-2F2, 813, 6A5, K2-4F2, 10.09, and 1B9 was associated with substantial loss of binding. However, K3-4C8, 3E1, AD2, AE8, and B5-B3 still effectively recognized the mutant virus (Fig. 2), even though the ability of several of these antibodies to neutralize S30 infectivity was substantially reduced (Table 3). None of the monoclonal antibodies, including the cognate B5-B3, showed reduced binding to S32 (data not shown).

DISCUSSION

The data reported here confirm and extend previous observations suggesting the existence of an immunodominant neutralization domain on the HAV capsid. Solid-phase competition studies indirectly suggest that the epitopes recognized by the 13 neutralizing monoclonal antibodies listed in Table 1 are closely clustered on the virion surface (Fig. 1). A combination of two of these monoclonal antibodies (K3-4C8 and B5-B3) competes effectively with polyclonal human postconvalescent antibody for attachment to the virus (8), suggesting that the immunodominance of the epitopes rec-

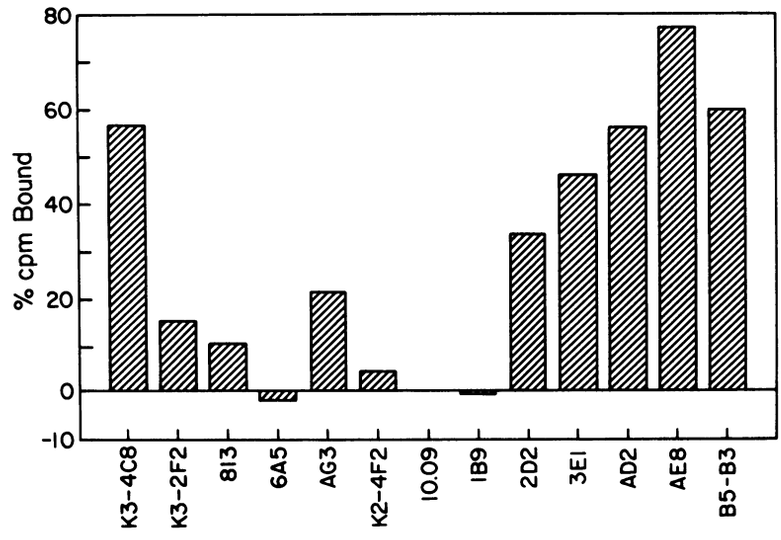

FIG. 2. Binding of neutralizing monoclonal antibodies to mutant S30 assessed by an indirect radioimmunoassay. Antibody binding to S30 is expressed as the percentage of antibody binding to equivalent amounts of parent HM175 virus in companion assays.

ognized by murine antibodies also extends to man. S30, a neutralization-escape mutant selected for resistance to antibody K2-4F2, is at least partially resistant to most other monoclonal antibodies (Table 3), providing further evidence for the immunodominance of this antigenic site. We now have partially localized this antigenic site by demonstrating the involvement of VP3. Aspartic acid-3070 contributes to this conformational antigen, as its replacement with histidine was the only mutation present in the capsid proteins of mutant S30.

This result was surprising, as previous studies have suggested a primary role for capsid protein VP1 in HAV antigenicity. Surface labeling studies with intact virus indicate that VP1 is the dominant surface protein of HAV (7). Furthermore, when Fab fragments from monoclonal antibodies 6A5 and 2D2 bound to and then were cross-linked to intact virus, subsequent separation of the capsid proteins by electrophoresis in denaturing gels suggested that these antibodies were predominantly associated with VP1 (15). Nonetheless, the participation of VP3 residues in the neutralization epitopes recognized by these same monoclonal antibodies (Fig. 2) is not completely unexpected, as in both poliovirus type 3 (PV3) and HRV14 there are conformationally dependent immunogenic neutralization sites that are formed by residues contributed by both VP3 and VP1 (10, 11). In addition, immunization of rats with either purified VP1 or VP3 from HAV has been shown to induce low levels of antibody capable of immunoprecipitating and neutralizing virus (25).

While sequence analysis of cloned genomic cDNA suggests that HAV shares a common genomic organization with other picornaviruses, there is very little homology between HAV and other picornaviruses at either the nucleotide or amino acid level. Indeed, a detailed comparison of known HAV genomic sequences with those of all other picornaviruses indicates that HAVs as a group are only distantly related to and are uniquely distant from any of the other picornaviruses (26). Nonetheless, it has been possible to align the capsid protein amino acid sequences of HAV with those

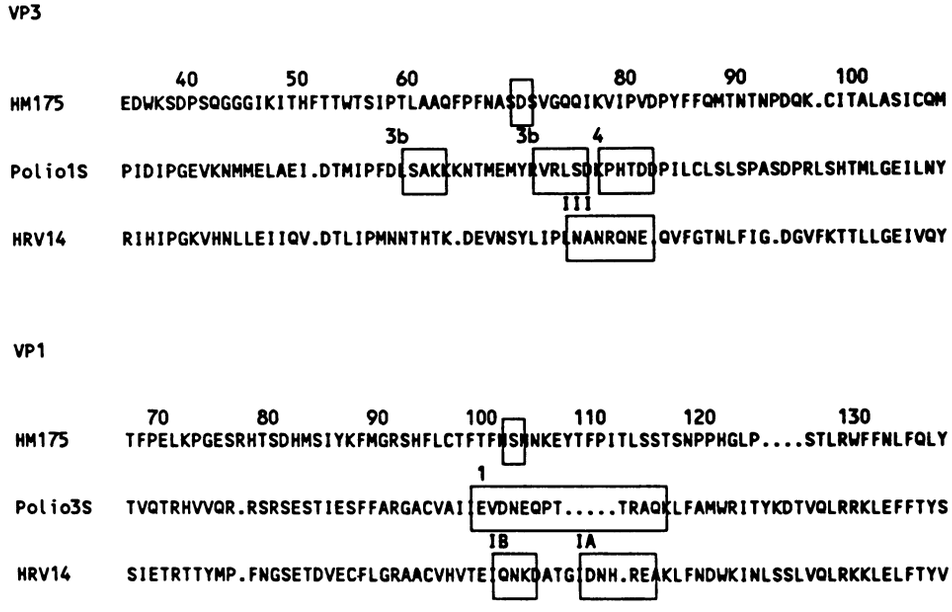

FIG. 3. (*Upper*) Amino acid residues recognized to contribute to neutralization immunogenic sites involving VP3 of HM175 strain HAV, Sabin strain PV1 (sites 3b and 4), and HRV14 (site NIm-III) (see text). The boxed residues are sites of mutation in escape mutants (with the exception of leucine-3072 of PV1, where no mutation has yet been identified). Sequences are aligned as described by Palmenberg (26), with HAV residues numbered at the top. (*Lower*) Similar alignments of partial amino acid sequences of VP1 from HM175 virus, Sabin PV3, and HRV14. Sites identified are PV3 site 1 and HRV14 sites NIm-IA and NIm-IB. The single-letter amino acid code is used throughout.

of other picornaviruses, based on a sophisticated search for similarities in the amino acid sequences (26). These alignments suggest that residue 3070 is part of an immunogenic site that is analogous to neutralization immunogenic sites known to involve VP3 of PV1 and PV3 [sites 3b or 4, terminology of Minor *et al.* (11, ‖)] (9, 11, 27) and HRV14 (site NIm-III) (10) (Fig. 3). Thus there appears to be substantial conservation of function as well as structure among these very distantly related viruses. These VP3 sites are discontinuous (conformational) structures in PV3 and HRV14, involving residues within VP3 and near the C terminus of VP1. The site identified in VP3 of HAV may involve analogous VP1 residues, which would explain the results of previous cross-linking studies with antibodies 6A5 and 2D2 (15).

Residue 1102 also aligns with recognized immunogenic sites in HRV14 and PV3 (10, 11) (Fig. 3) and is the only site of mutation in the capsid proteins of mutant S32. However, the finding that mutations at residues 1102 and 3070 are functionally related is confusing. At least partial resistance to antibodies B5-B3, 3E1, 2D2, 10.09, and possibly 6A5 is conferred by mutations at either residue 1102 or residue 3070 (Table 3). The analogous residues are distant from each other in the three-dimensional structures of poliovirus and HRV14 and contribute to functionally independent antigenic sites (9–11, 26). In contrast, the present data suggest that these residues are relatively closely positioned on the HAV capsid. A less likely alternative is that neutralization resistance might be caused by capsid mutations occurring at a distance from the primary antibody binding site.

Although the neutralization resistance of mutant S30, selected for resistance to K2-4F2, extended to most of the other monoclonal antibodies, many of these antibodies continued to bind to the mutant virus despite lack of efficient neutralization (compare Table 3 and Fig. 2). This may reflect a requirement for bivalent binding of many monoclonal antibodies for neutralization to occur, as demonstrated with poliovirus (28). Relatively minor perturbations in the conformation of epitopes could continue to permit monovalent binding of antibody, but lead to unfavorable binding energies with respect to bivalent attachment and associated changes in the capsid conformation that might be required for neutralization.

We conclude from these studies that residue 3070 contributes to the immunodominant HAV antigenic site recognized by most available monoclonal antibodies. Recognition of the involvement of VP3 is important for the development of a HAV vaccine. Additional mapping of capsid protein amino acid residues contributing to this site may assist in the development of vaccine immunogens by various biotechnological approaches.

Note Added in Proof. Since submission of this article, we have completed sequencing the P1 region of 43c12 virus. One additional mutation was found: methionine to valine at residue 1276.

‖Ferguson, M., Magrath, D. I. & Minor, P. D. (1987) Antigenic Variation Among Poliovirus Strains, 5th Meeting of the European Group of Molecular Biology of Picornaviruses (abstr.).

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