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Antigenic Structure of Human Hepatitis A Virus Defined by Analysis of Escape Mutants Selected against Murine Monoclonal Antibodies

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We examined the antigenic structure of human hepatitis A virus (HAV) by characterizing a series of 21 murine monoclonal-antibody-resistant neutralization escape mutants derived from the HM175 virus strain. The escape phenotype of each mutant was associated with reduced antibody binding in radioimmunofocus assays. Neutralization escape mutations were identified at the Asp-70 and Gln-74 residues of the capsid protein VP3, as well as at Ser-102, Val-171, Ala-176, and Lys-221 of VP1. With the exception of the Lys-221 mutants, substantial cross-resistance was evident among escape mutants tested against a panel of 22 neutralizing monoclonal antibodies, suggesting that the involved residues contribute to epitopes composing a single antigenic site. As mutations at one or more of these residues conferred resistance to 20 of 22 murine antibodies, this site appears to be immunodominant in the mouse. However, multiple mutants selected independently against any one monoclonal antibody had mutations at only one or, at the most, two amino acid residues within the capsid proteins, confirming that there are multiple epitopes within this antigenic site and suggesting that single-amino-acid residues contributing to these epitopes may play key roles in the binding of individual antibodies. A second, potentially independent antigenic site was identified by three escape mutants with different substitutions at Lys-221 of VP1. These mutants were resistant only to antibody H7C27, while H7C27 effectively neutralized all other escape mutants. These data support the existence of an immunodominant neutralization site in the antigenic structure of hepatitis A virus which involves residues of VP3 and VP1 and a second, potentially independent site involving residue 221 of VP1.

X-ray crystallographic determinations of virus structures have contributed substantially to current understanding of the structural organization and function of picornaviruses (1, 15, 26, 32). Combined with the analysis of neutralization escape mutants selected for resistance to monoclonal antibodies, structural studies have provided a uniquely detailed view of the antigenic features of these viruses (6, 28, 32). However, technical difficulties have severely hampered such studies with human hepatitis A virus (HAV), a medically important virus now classified as the type species of the genus *Hepatovirus* within the family *Picornaviridae* (14). The replication cycle of HAV in cell culture is relatively slow, and yields of virus are considerably lower than those obtained with most other picornaviruses (4). Thus, production of the quantities of purified virus that are required for crystallographic studies represents a daunting task. In addition, HAV replication is usually nonlytic and neutralization of HAV in vitro is often relatively inefficient (19), making the isolation and characterization of neutralization escape mutants both tedious and difficult.

Nonetheless, some information concerning the antigenic structure of HAV has been obtained from studies with a very limited number of escape mutants. By repetitive cycles of neutralization and amplification of virus in the presence of murine monoclonal antibodies, we previously isolated a small series of escape mutants (31, 34). Neutralization studies with these escape mutants and a related panel of monoclonal antibodies suggested that most murine antibodies recognize a dominant antigenic site on the virus capsid. These results were further supported by studies examining competition between various monoclonal antibodies for binding to the surface of the virus capsid (16, 31, 34). Sequencing studies indicated that the Asp-70 residue of capsid protein VP3 (Asp 3-070) (in this paper, we use four-digit nomenclature to describe specific amino acid residues; i.e., "y-nnn," in which "y" represents the capsid protein [VP1, VP2, or VP3] and "nnn" represents the residue number from the proposed amino terminus [9]) plays a critical role in forming this antigenic site, while a lesser contribution is made by Ser 1-102 (31). These residues are well conserved among human HAV strains, consistent with the highly conserved antigenic characteristics of this virus. Subsequent mutagenesis studies with infectious cDNA have confirmed the importance of Asp 3-070 in the antigenic structure of HAV (11).

In this report, we describe the isolation and characterization of an expanded series of HAV escape mutants that resist neutralization by one or more murine monoclonal antibodies. Results of these studies are consistent with a critical role for Asp 3-070 in the structure of an immunodominant antigenic site, but they also document the contribution of additional residues of VP1 to this domain and suggest the existence of a second, potentially independent antigenic site involving other residues of VP1. These results are of interest because they shed additional light on the structure of this virus and thus allow further comparisons of HAV with other picornaviruses. A better understanding of the antigenic structure of HAV is also relevant to the rational design of new vaccines, which are needed for improved control of type A viral hepatitis in many human populations (33).

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MATERIALS AND METHODS

Virus and cells. Virus was propagated in continuous African green monkey kidney (BS-C-1) cells, which were grown as monolayers in Eagle's minimal essential medium with Earle's salts (EMEM) supplemented with 100 mM glutamine, streptomycin (100 μg/ml), penicillin (100 U/ml), and 2 to 10% fetal bovine serum (4). Neutralization escape mutants were selected from a clonally isolated, rapidly replicating, cytopathic (RR/CPE$^+$) variant of the HM175 strain of HAV, HM175/18f virus (25), as described in detail below. HM175/18f is highly adapted to growth in cell culture and contains two capsid protein mutations, while its parent, HM175/p16, is an RR/CPE$^-$ virus which has undergone only 16 passages in cell culture. These mutations, which include substitutions of Thr 3-091 with Lys and Ser 1-271 with Pro, do not alter the antigenicity of HM175/18f virus in solid-phase immunoassays utilizing a panel of murine monoclonal antibodies (25). Previously described escape mutants (S30 and S32) were isolated from HM175/p16 virus (34), while HM175/43c is a spontaneous RR/CPE$^+$ neutralization escape mutant recovered from persistently infected cells in the absence of any antibody-related selective pressure (25). This virus lacks the two capsid mutations of HM175/18f but has three other amino acid substitutions in the capsid proteins as described previously (25, 31). HM175/30M is another spontaneous escape mutant which was initially identified in an epitope-specific (K24F2) radioimmunofocus assay (see below) of cells infected with HM175/18f virus in the absence of any selective antibody pressure, and it was clonally isolated as described below.

Monoclonal antibodies to HAV. Hybridoma cells secreting antibodies 1.193 and 1.134 were selected from the fusion products of P3-8.653 cells (American Type Culture Collection) and spleen cells harvested from BALB/c mice immunized with gradient-purified HM175/18f virus. Antibody-secreting hybridomas were identified by testing supernatant fluids of cultures in an indirect radioimmunoassay. In this assay, 96-well polyvinylchloride microplates were coated with goat antibody to mouse immunoglobulins (immunoglobulin G [IgG], IgM, and IgA) and then loaded with supernatant fluids from hybridoma cultures. Virus-specific antibodies captured to the solid phase were identified by their ability to bind virus present in crude lysates of infected cells; immobilized viral antigen was subsequently detected by incubation with ^{125}I-labelled human polyclonal (JC) antibody to HAV. Other monoclonal antibodies were provided as gifts from multiple investigators or were purchased from Commonwealth Serum Laboratories, Melbourne, Australia (see Table 1). Antibody preparations utilized in these studies included hybridoma culture supernatants, purified immunoglobulins, and mouse ascitic fluids.

When the immunoglobulin class of a monoclonal antibody was not known from its original source, antibodies present in hybridoma culture fluids were typed by using an ImmunoSelect kit purchased from Bethesda Research Laboratories, Bethesda, Md. Monoclonal antibodies present in ascitic fluids were typed by using a modification of this procedure. Antibodies in ascitic fluids were captured onto the wells of 96-well plates with immunoglobulin subclass-specific antibodies (ImmunoSelect; Bethesda Research Laboratories), and antibodies specific for HAV were identified by the sequential addition of virus-infected cell lysates and ^{125}I-labelled human anti-HAV IgG. For use in immunoassays, monoclonal antibodies were partially purified by precipitation with 50% ammonium sulfate and then dialyzed against phosphate-buffered saline and subjected to chloramine-T-mediated labelling with ^{125}I, as described previously (22).

Virus neutralization. Neutralization of HAV was assessed in radioimmunofocus-reduction assays as described previously (18). This method is similar to conventional plaque-reduction neutralization assays for viruses but involves staining of acetone-fixed cell sheets with ^{125}I-labelled anti-HAV, followed by autoradiography, for visualization of HAV replication foci. Despite the RR/CPE$^+$ phenotype of HM175/18f virus, the radioimmunofocus assay remains the most reliable method for determining the infectious titer of this virus.

Solid-phase radioimmunoassays. Solid-phase radioimmunoassays for HAV and anti-HAV antibodies were carried out as described previously (24). Assays determining the ability of individual monoclonal antibodies to compete with polyclonal or other monoclonal antibodies for binding to HAV have also been described elsewhere (34). In these assays, fivefold dilution series of each monoclonal antibody were tested for activity in blocking the binding of ^{125}I-labelled human polyclonal (JC) or similarly labelled monoclonal K34C8 and B5B3 antibodies to virus which had been captured onto a solid-phase support by human polyclonal antibody. The competing unlabelled antibody was present in substantial molar excess over the radiolabelled antibody, such that antibody concentration was not limiting with respect to blocking activity.

Selection of neutralization escape mutants. Attempts to neutralize HAV almost always result in substantial nonspecific nonneutralized fractions (19), even after vigorous attempts to eliminate virus aggregation. Thus, we subjected virus to repeated cycles of neutralization followed by amplification in the presence of murine monoclonal antibodies, using a general approach that we have described previously (34). However, to facilitate the process of mutant selection, we utilized an epitope-specific radioimmunofocus assay technique (21, 25) following the first three to four cycles of neutralization and virus amplification, as shown in Fig. 1. To reduce aggregation, HM175/18f virus was brought to 0.1% sodium dodecyl sulfate (SDS) and held at 37°C for 30 min. This was followed by extraction with an equal volume of chloroform and sonication for 3 min. An aliquot containing approximately 10^7 radioimmunofocus-forming units was neutralized by incubation with a monoclonal antibody (2D2, AD2, AE8, or H7C27) overnight at 4°C followed by incubation for 1 h at 35.5°C prior to adsorption to nearly confluent monolayers of BS-C-1 cells in replicate 25-cm^2 tissue culture flasks (three or six flasks, labelled A, B, and C, etc.) for 2 h at 35.5°C. The concentration of each monoclonal antibody was 10- to 1,000-fold greater than that required to achieve 50% neutralization of virus. The inoculum was removed, and the cell sheet was washed three times with EMEM containing 2% fetal bovine serum. The cells were refed with 5 ml of maintenance medium containing 1/10th the original concentration of monoclonal antibody and incubated at 35.5°C. After 7 days of incubation, the medium was removed and used as a source of virus for the second cycle of neutralization-amplification (Fig. 1). This medium was extracted once with an equal volume of chloroform prior to the addition of fresh antibody for the subsequent neutralization cycle.

After three to four cycles of neutralization-amplification, the neutralization resistance of virus present in cell culture supernatant fluids was assessed in an epitope-specific radioimmunofocus assay (21). This virus was treated with SDS and neutralized with monoclonal antibody as described above, and 10-fold dilutions of neutralized virus were inoc-

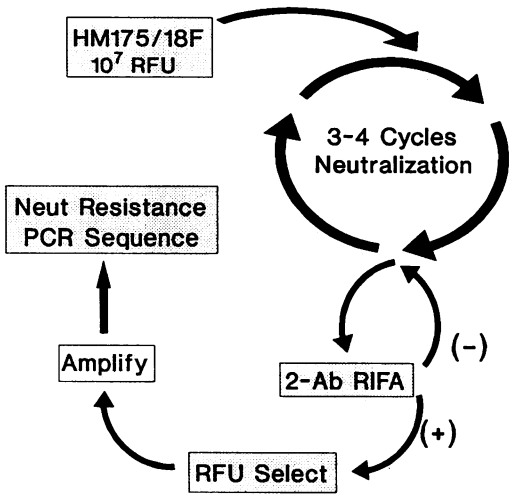

FIG. 1. Approach taken for the isolation of neutralization escape variants of HM175/18f virus. For each monoclonal antibody, three (AD2, AE8, and H7C27) or six (2D2) replicate cultures of BS-C-1 cells were inoculated with neutralized virus. Virus harvested from supernatant fluids of each culture was carried through three to four cycles of neutralization-amplification in BS-C-1 cells with antibody maintained in the medium, and progeny were examined for resistant virus clones by an epitope-specific (double-antibody) radioimmunofocus assay (2-Ab RIFA) employing the cognate monoclonal antibody. At least two neutralization-resistant virus clones were selected from each neutralization-amplification culture series and amplified in BS-C-1 cells for determination of the neutralization resistance phenotype and PCR-based sequence analysis. RFU, radioimmunofocus-forming units.

ulated onto nearly confluent BS-C-1 cell monolayers in 60-mm-diameter polystyrene petri dishes. After 2 h of virus adsorption, the cell sheets were overlaid with medium containing 0.5% SeaKem agarose. Following 7 days of incubation at 35.5°C in a 5% CO_2 atmosphere, the overlay was carefully removed and placed at 4°C and the cells were fixed with 80% acetone and stained with the cognate ^{125}I-labelled monoclonal antibody (20). Following autoradiographic exposure, the cell sheet was counterstained with ^{125}I-labelled polyclonal human convalescent antibody (JC) to HAV and a second autoradiographic exposure was obtained. This epitope-specific radioimmunofocus assay procedure allowed the overall neutralization resistance of the virus harvest to be assessed by comparison of virus titers before and after neutralization. However, the double-antibody staining procedure also identified individual virus clones which no longer bound the monoclonal antibody of interest (21, 25). At least two monoclonal antibody-resistant clones were recovered from each original neutralization-amplification culture series (A, B, and C, etc.) by sampling agarose overlays in the regions overlying selected radioimmunofoci. Agarose plugs removed from the overlay were processed for virus recovery as described previously (23). When the epitope-specific radioimmunofocus assay results indicated that the virus harvest was a mixture of resistant and nonresistant viruses, the resistant (mutant) clones were plaque purified twice by this procedure. Otherwise, mutant viruses were plaque purified only once prior to amplification. Virus recovered from overlays was neutralized overnight with the cognate antibody and amplified in 25-cm^2 flasks in the presence of the monoclonal antibody to prepare a virus seed for determination of neutralization resistance to the complete panel of antibodies and for sequencing of virion RNA. If no escape mutants were identified in the epitope-specific radioimmunofocus assay, the virus was subjected to an additional three neutralization-amplification cycles, as described above (Fig. 1).

Analysis of neutralization resistance phenotypes. Escape mutant harvests were prepared from supernatant fluids of 25-cm^2 cell culture flasks 7 days after inoculation of virus, and the virus titer was determined by radioimmunofocus assay. A standard inoculum of each mutant virus (approximately 400 radioimmunofocus-forming units/ml) prepared in medium containing 2% fetal bovine serum was mixed with an equal volume of antibody for neutralization as described above, and the residual virus titer was determined by radioimmunofocus assay (18). We defined neutralization resistance as <30% neutralization, partial neutralization resistance as 30 to 60% neutralization, and neutralization sensitivity as >60% neutralization. For comparison, a parental HM175/18f virus control was included for each monoclonal antibody tested in each neutralization assay.

Nucleotide sequence of escape mutants. The partial nucleotide sequence of clonally isolated escape mutants was determined by dideoxynucleotide sequence analysis of the products of an antigen capture-polymerase chain reaction (PCR) method described previously (17). Polyclonal human antibody (JC) was utilized for capture of virus. Positive (+)- and negative (−)-strand oligodeoxynucleotide primer pairs utilized for reverse transcription and PCR amplification of cDNA included (among others) (i) +560/−1501, (ii) +1561/−1771, (iii) +2646/−3192, (iv) +1561/−2037, (v) +2392/−2698, (vi) +1308/−1771, (vii) +1998/−2556, and (viii) +1308/−2556, where the number indicates the position of the most 5′ base within the positive strand represented by the oligonucleotide (wild-type HM175 numbering) (9). PCR amplification products were subjected to agarose gel electrophoresis and the specific band was excised and extracted with phenol-chloroform. Multiple positive-strand and negative-strand primers were utilized for ^{35}S-dideoxynucleotide sequencing of the double-stranded cDNA. Primer sequences are available upon request.

RESULTS

Characterization of murine monoclonal antibodies to HAV. We assembled a panel of 24 murine monoclonal antibodies raised against eight different strains of human HAV in nine different laboratories. A total of 4 of these antibodies were of the IgM isotype, 1 was IgA, and the remaining 19 were IgG (Table 1). Fifteen IgG antibodies were of the subclass IgG2A, while two antibodies were IgG3, one was IgG2B, and one was IgG1. A total of 15 of 16 antibodies for which the light-chain type was determined were of the κ type.

Serial dilutions of monoclonal antibodies were tested for their ability to block the binding of polyclonal human convalescent antibody to HAV in a solid-phase radioimmunoassay (Table 1). Eighteen of these antibodies were capable of blocking polyclonal antibody binding by ≥50%. In addition, we characterized each antibody with respect to its ability to compete against two individual reference monoclonal anti-HAV antibodies (K34C8 and B5B3) for binding to HAV in solid-phase immunoassays (Fig. 2). We previously demonstrated that these two antibodies do not compete with each other for binding to the virus capsid and that a combination of these two antibodies is capable of blocking the binding of polyclonal human antibody almost completely (31, 34). As shown in Fig. 2, measurement of K34C8- and B5B3-blocking

TABLE 1. Murine monoclonal antibodies to HAV

Antibody	Immunoglobulin class	Virus strain	Source[a]	Maximum % competition vs pcAB[b]	Neutralization[c]	
					%	Titer
K34C8	IgG2A(κ)	HM790	g	47	92	>5.0
1.193	IgA(κ)	HM175	h	74	98	4.3
K24F2	IgG2A	HM790	g	59	100	>5.0
H10C29	IgG3	GBM	b	39	91	
K32F2	IgG2A	HM790	g		100	>5.0
813	IgG3(κ)	CF53	a	54	97	
141C19	IgG2A(κ)	GBM	b	60	99	
6A5	IgG2A	CR326	e	62	91	6.0
AG3	IgG2A(κ)	S841	f	69	100	3.9
1009	IgG1(κ)	CF53	a	51	100	
H14C42	IgM(λ)	GBM	b	50	96	
1B9	IgG2A	CR326	e	40	99	3.5
2D2	IgG2A(κ)	CR326	e	65	94	5.5
3E1	IgG2A	CR326	e	65	86	4.0
B5B3	IgG2A	KMW1	i	60	98	5.1
H80C25	IgG2A(κ)	GBM	b	65	85	
AD2	IgG2A(κ)	S841	f	87	95	3.4
7E7	IgG2A(κ)	GBM	c	77	96	3.6
AE8	IgM(κ)	S841	f	66	92	5.3
H29C26	IgG2A(κ)	GBM	b	71	97	
H7C27	IgG2B(κ)	GBM	b	70	94	3.7
4E7	IgM(κ)	GBM	c	73	97	
1.134	IgM(κ)	HM175	h	13	<50	<1.0
LSH-14H	IgG2A	LSH/S	d	36	<50	<1.0

[a] Sources: a, D. Crevat, Clonatec, Paris, France (12); b, R. Decker, Abbott Laboratories, North Chicago, Ill. (13); c, B. Flehmig, University of Tubingen, Tubingen, Germany; d, H. Garelick, London School of Tropical Medicine and Hygiene, London, United Kingdom; e, J. Hughes, Merck Sharp and Dohme Research Laboratories, West Point, Pa. (16); f, C. Li, Sichuan Health and Anti-Epidemic Station, China; g, Commonwealth Serum Laboratories, Melbourne, Australia (27); h, L.-H. Ping, University of North Carolina, Chapel Hill, N.C. (this paper); i, R. Tedder, Middlesex Hospital, London, United Kingdom.

[b] Maximum percent competition (at highest antibody concentration) against polyclonal human antibody (JC) in a solid-phase radioimmunoassay employing HM175/18f antigen (34).

[c] Percent neutralization of HM175/18f virus achieved at working concentration; titer is the $\log_{10}$ dilution of ascitic fluid or hybridoma culture supernatant fluid resulting in 50% neutralization of HAV (HM175/18f or HM175/p16) (34).

activity is a useful approach to characterizing monoclonal antibodies to HAV. The results of these solid-phase radioimmunoassays allowed us to categorize the antibodies into the following five groups: group A, which almost completely blocks the binding of K34C8 but does not reduce (and often enhances) the binding of B5B3; group B, which has significantly greater blocking activity against K34C8 than B5B3; group C, which has strong and roughly equivalent blocking activity against both K34C8 and B5B3; group D, which has greater blocking activity against B5B3 than K34C8; and group E, which includes only B5B3 and which has no appreciable activity in blocking the binding of K34C8 (Fig. 2).

All but two antibodies (1.134 and LSH-14H) had substantial neutralization activity against HM175/18f virus (≥85% reduction in infectious-virus titer) (Table 1). The two monoclonal antibody preparations with low neutralizing activity (<50% neutralization), one of which was IgM(κ) and the other of which was IgG2A, were excluded from further analysis.

Isolation of neutralization escape mutants. We have shown previously that HM175 virus mutants with His or Ala substitutions at Asp 3-070 resist neutralization by many murine monoclonal antibodies (see mutants S30 and 43c in Fig. 3) (31). Additionally, an escape mutant having a Leu substitution at Ser 1-102 (mutant S32) demonstrated partial resistance to several antibodies. As mutations at either of these residues confer resistance to several antibodies (2D2, B5B3, and 3E1), these data suggested that residues 3-070 and 1-102 both contribute to a single, dominant neutralization site on the capsid surface (31). To test this hypothesis, we determined whether multiple, independently selected mutants capable of escaping neutralization by the monoclonal antibody 2D2 would demonstrate mutations in both VP3 and VP1.

The isolation of escape mutants resistant to 2D2 required between three and six neutralization-amplification cycles, as indicated in Table 2. Mutant viruses isolated from each of six independent neutralization-amplification culture series failed to bind 2D2 in an epitope-specific radioimmunofocus assay (data not shown), but each was detected with radiolabelled polyclonal human antibody. Two mutants were clonally isolated from each of the six culture series (A through F). Both clones from five of the six culture series had identical mutations within the nucleotide sequence (and were thus considered to be sibling clones), while the two mutants from the remaining culture series (series B) each had unique mutations. Thus, we isolated a total of seven independent mutants that were resistant to 2D2. Each of these seven mutants had closely spaced mutations within VP3 (3-070 or 3-074) (Table 2). Four mutants had an Asn substitution at Asp 3-070, while two had a Tyr substitution at the same residue and one had an Arg substitution at Gln 3-074. As we have confirmed previously the involvement of residue 3-070 in the dominant antigenic site of HAV, we carried out only limited sequencing of these VP3 mutants (Fig. 4).

We selected three other antibodies (AD2, AE8, and H7C27) for the generation of additional escape mutants because of evidence that these antibodies are directed against epitopes that are distinct from that bound by 2D2. Two of these antibodies have monoclonal antibody competition profiles in solid-phase radioimmunoassays that are substantially different from that of 2D2 (AD2 and AE8 are in group D, while 2D2 is in group B) (Fig. 2). The competition profile of the third antibody, H7C27 (group C), is relatively unique in that it has approximately equal blocking activity against K34C8 and B5B3 but is unable to block the binding of either more than about 70%. In addition, while resistance to AD2 was conferred by a His substitution at Asp 3-070 (mutant S30), AE8 and H7C27 were able to neutralize previously isolated 3-070 and 1-102 mutants (Fig. 3).

For both AD2 and AE8, we selected independent mutants from three parallel neutralization-amplification culture series. These escape mutants generally required a higher number of neutralization-amplification cycles for isolation than escape mutants selected against 2D2, as resistant virus was identified in only one of six culture series after four cycles (series AE8-A), compared with five of six culture series carried out in the presence of 2D2 (Table 2). While the neutralization titer of the AD2 ascitic fluid (50% neutralization endpoint between 10^{-3} and 10^{-4}) was significantly lower than that of 2D2 (between 10^{-5} and 10^{-6}), this was not the case with AE8 (also between 10^{-5} and 10^{-6}). Thus, the greater number of neutralization-amplification cycles required with AE8 and AD2 could be only partially related to the lower level of neutralizing activity of these antibodies. In addition to their lack of neutralization (≤10%) with the cognate antibody, the AD2 and AE8 mutants failed to bind

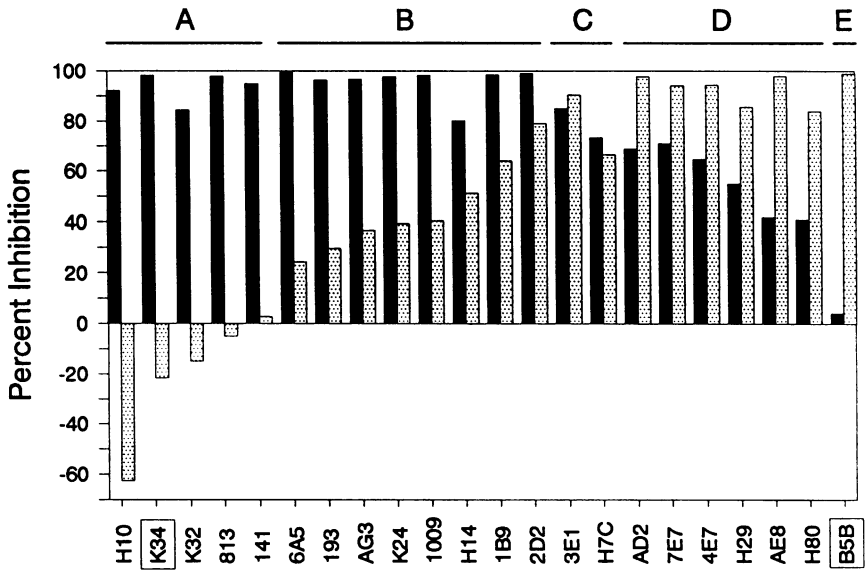

FIG. 2. Results of solid-phase radioimmunoassays examining the ability of monoclonal antibodies (labelled according to the first three or four characters of each complete antibody designation shown in Table 1) to compete with radiolabelled antibodies K34C8 (solid columns) or B5B3 (stippled columns) for binding to immobilized viral antigen. Monoclonal antibodies were characterized as belonging to one of five different monoclonal antibody competition profile groups (A through E), as shown at the top of the figure. K34C8 and B5B3 antibodies utilized as labelled reagents are boxed at the bottom of the figure.

this antibody in epitope-specific radioimmunofocus assays (data not shown).

Two mutant viruses were clonally isolated from each neutralization-amplification culture series. Both clonal variants isolated from each of the three culture series selected for AE8 resistance had a Glu substitution at Val 1-171 (Table 2). The same substitution was also present in both clonal isolates from one culture series selected for AD2 resistance (series AD2-A), while an Asp substitution at Ala 1-176 was found in both clonal isolates from each of the other two AD2 neutralization-amplification culture series (Table 2). As mutations at 1-171 and 1-176 of HAV have not been associated previously with neutralization escape, we determined the complete P1 sequence of two representative mutants, 11C (Glu 1-171 selected against AE8) and 20D (Asp 1-176 selected against AD2) (Fig. 4). In both cases, no other nonsilent mutations from the HM175/18f parent nucleotide sequence were identified within the P1 region. These results thus indicate that AD2 and AE8 recognize closely spaced epitopes involving residues 171 and 176 of VP1.

We also selected mutants against antibody H7C27 in three separate neutralization-amplification culture series. In each case, escape mutants which failed to bind H7C27 in an epitope-specific radioimmunofocus assay were present after four cycles of neutralization-amplification (Table 2). Altogether, five escape mutants were clonally isolated from the three culture series and amplified for further analysis. We found that each of these escape mutants had undergone mutation within the Lys 1-221 codon (Table 2). One clonal isolate from culture series A had a Glu 1-221 substitution due

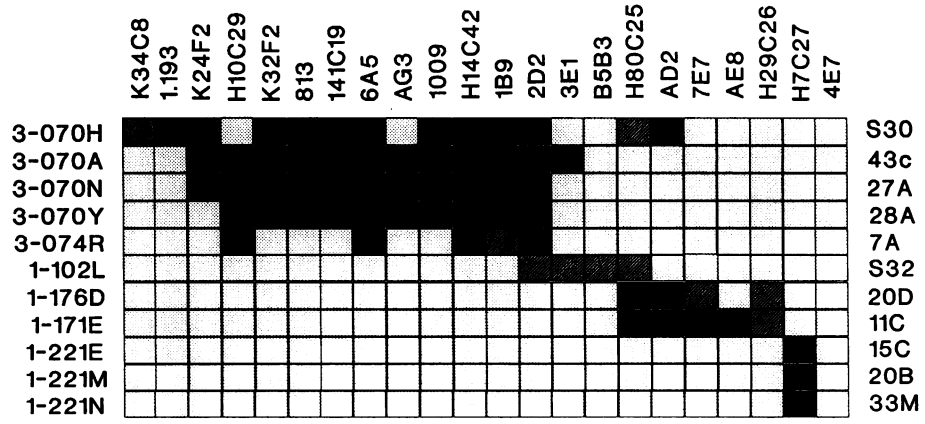

FIG. 3. Cross-resistance of neutralization escape mutants to a panel of 22 neutralizing murine monoclonal antibodies. Eleven mutants (listed to the right) representing unique amino acid substitutions within the capsid proteins (listed to the left) were characterized as resistant (<30% neutralization) (solid boxes), partially resistant (30 to 60% neutralization) (hatched boxes), or sensitive (>60% neutralization) (stippled boxes) to each antibody.

TABLE 2. Neutralization escape mutants derived from the HM175 strain of HAV[a]

Antibody	Mutant	Series[b]	Cycle[c]	Base change at position	Substitution at:					
					Asp 3-070	Gln 3-074	Ser 1-102	Val 1-171	Ala 1-176	Lys 1-221
K24F2	S30	A	3	1677	His					
B5B3	S32	A	3	2512			Leu			
	43c			1678[d]	Ala					
	30M			1677	Asn					
	27A	A	6	1677	Asn					
	18A	B	3	1677	Asn					
	3A	B	4	1677	Tyr					
2D2	7A	C	4	1690		Arg				
	22A	D	3	1677	Asn					
	28A	E	3	1677	Tyr					
	34B	F	3	1677	Asn					
	4C	A	6	2719				Glu		
AD2	16C	B	6	2734					Asp	
	20D	C	6	2734					Asp	
	40A	A	4	2719				Glu		
AE8	11C	B	6	2719				Glu		
	47M2	C	6	2719				Glu		
	15C	A	4	2868						Glu
H7C27	20B	B	4	2869						Met
	33M	C	4	2870						Asn
	24B	C	4	2870						Asn

[a] For completeness, two previously isolated mutants (S30 and S32) (31) and two spontaneous RR/CPE$^+$ mutants (43c and 30M) (25) are included.
[b] Culture-selection series from which the mutant was isolated.
[c] Neutralization-amplification cycle at which the mutant was identified (see Materials and Methods).
[d] Also at positions 2797 and 3033.

to a base change at position 2868 (HM175 wild-type numbering), while two mutants from culture series B had a Met 1-221 substitution due to a base change at position 2869. On the other hand, while both mutants (33M and 24B) isolated from culture series C had Asn 1-221 substitutions, these were due to different base changes at the third codon position, 2870 (Table 2). Thus, four unique H7C27-resistant mutants were isolated, each with amino acid replacements at residue 1-221. As mutations at this residue have not been associated previously with neutralization escape, we determined the complete P1 nucleotide sequence of mutant 33M (Asn 1-221). We found no other nonsilent mutation from the nucleotide sequence of the HM175/18f parent (Fig. 4).

Cross-resistance of escape mutants to other monoclonal antibodies. We characterized the neutralization resistance phenotype of escape mutants by testing each for resistance to each of the 22 monoclonal antibodies which we found to have significant neutralizing activity against the parent HM175/18f virus (Table 1). For these studies, we included mutant viruses with unique amino acid substitutions, selecting only one representative mutant when multiple mutants with the same amino acid replacement had been isolated. These results, in addition to neutralization results obtained with three previously isolated mutants, S32, S30 and 43c, are summarized in Fig. 3 (31). Neutralization resistance was determined in radioimmunofocus reduction assays with concentrations of antibody which were capable of effectively neutralizing an HM175/18f virus inoculum in parallel assays (see Materials and Methods).

Mutants with amino acid substitutions at Asp 3-070 demonstrated partial or complete neutralization resistance against 16 of the 22 antibodies (Fig. 3). In addition, although

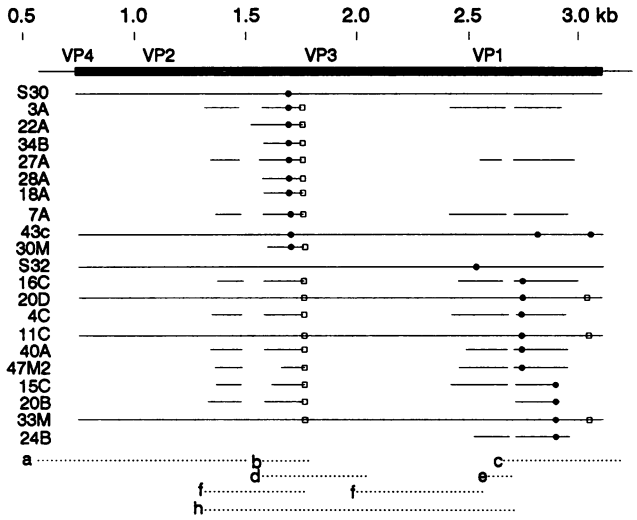

FIG. 4. Genomic regions sequenced within individual neutralization escape mutants (listed at the left). The map of the P1 region of the HM175 virus genome is shown at the top, with the location of bases encoding each capsid protein indicated. Horizontal lines indicate regions for which the nucleotide sequence was determined. The complete P1 sequence of mutants S30, S32, 43c, 20D, 11C, and 33M has been determined. ●, nonsilent mutations within the nucleotide sequence; □, nonsilent mutations (from the wild-type virus) present in the parent HM175/18f virus from which all mutants other than S30, S32, and 43c were derived. Silent mutations are not shown. The location of cDNA segments amplified by PCR for sequence determination is shown at the bottom, with labels referring to the primer sets described in Materials and Methods. Only one virus for which two virus clones with identical mutations were recovered from the same culture series is shown.

mutant S32 (Leu 1-102, selected against B5B3) was sensitive to neutralization by K24F2 according to the stringent criteria for neutralization resistance employed in this study (<30% neutralization for resistance and 30 to 60% neutralization for partial resistance), we have previously shown in replicate experiments that S32 is less susceptible to neutralization by this antibody than is its parent, HM175/p16 virus (31). The broadest resistance was observed with mutants which had either His or Ala replacements at residue 3-070 (previously isolated mutants S30 and 43c), while mutant 7A, which has an Arg substitution at Gln 3-074, had a considerably narrower resistance profile (Fig. 3). These results indicate that residues Asp 3-070 and Gln 3-074 contribute to an important neutralizing antigenic domain which is dominant in the murine antibody response.

Mutants 20D and 11C, which were selected against antibodies AD2 and AE8, respectively, were also resistant to H80C25, 7E7, and H29C26 (partial resistance only) (Fig. 3). As resistance to AD2 and H80C25 was also conferred by a His substitution at Asp 3-070, these results indicate that Val 1-171 and Ala 1-176 (sites of mutations in 11C and 20D) contribute to this same immunodominant antigenic site. This was further confirmed by the resistance pattern of mutant S32. This mutant has a Leu replacement at Ser 1-102 (31) and was partially resistant to antibodies 2D2, 3E1, and H80C25, as well as B5B3, against which it was originally selected (34). Thus, the dominant antigenic site of HAV includes contributions from Asp 3-070, Gln 3-074, Ser 1-102, Val 1-171, and Ala 1-176. A total of 20 of 22 murine neutralizing antibodies may be related to this site by resistance due to mutations at one or more of these residues (Fig. 3). This site is complex, however, and contains multiple epitopes, as resistance to most individual antibodies was mediated by mutations within only one or two putative protein loops on the surface of the capsid. Antibody H80C25 represents an exception to this statement, however, as partial or complete resistance was mediated by substitutions within the three putative protein loops containing residue 3-070, 1-102, or 1-171 and 1-176.

All three escape mutants with unique substitutions at Lys 1-221 were resistant only to antibody H7C27, the antibody against which they had been selected (Fig. 3). Conversely, this antibody effectively neutralized all of the remaining escape mutants. Thus, the available data suggest that the antigenic site identified by mutations at Lys 1-221 may be functionally distinct from the immunodominant site described above. Similarly, antibody 4E7 neutralized each of the escape mutants, including those with mutations at Lys 1-221 (Fig. 3). Thus, the antigenic site against which 4E7 is directed remains undefined and may possibly represent yet a third, functionally independent site.

DISCUSSION

Although the structures of the capsids of representative members of each of the four other picornaviral genera have been determined by X-ray crystallography (1, 15, 26, 32), this has not yet been accomplished for HAV because of the difficulties inherent in producing sufficient quantities of virus for such studies. However, it is likely that the structure of HAV resembles that determined for other picornaviruses in terms of the general orientation of the individual proteins within the capsid and the presence of an 8-stranded, antiparallel β-barrel structural motif with flanking loop regions which is shared by the capsid proteins of other picornaviruses. Moreover, the approximate locations of the β-strands and intervening loop sequences of HAV may be inferred by sophisticated methods of examination of alignments of the amino acid sequences of HAV with those of other picornaviruses (29).

The data we report in this paper generally support the existence of two independent antigenic sites on the HAV capsid. One of these sites can be considered immunodominant in the mouse, as most murine neutralizing monoclonal antibodies are directed against epitopes within it. This immunodominant site is defined by mutations at residues 3-070 and 3-074, as well as at residues 1-102, 1-171, and 1-176. These latter residues align approximately with the B-C and E-F loops of VP1 of Mengo virus (MV), a murine cardiovirus, while residues 3-070 and 3-074 of HAV align with the VP3 "knob" of MV (29, 26). Although there is little relatedness evident in the amino acid sequences of the capsid proteins of HAV and MV (35), these two picornaviruses are similar in terms of the probable location of the primary polyprotein cleavage event (3), the probable presence of a leader (L) peptide (8), and (perhaps most significant) conserved RNA structural motifs within the 5′ nontranslated region of the genome (7). In contrast, the enteroviruses (among which HAV was classified previously) and the rhinoviruses differ from the cardioviruses and the hepatoviruses in each of these three respects. Consistent with this structural alignment, the predicted VP3 knob figures prominently in the antigenic structure of HAV, as it does in MV (6). However, functional relatedness and close structural proximity of the VP1 B-C and E-F loops to the VP3 knob in HAV are suggested by the pattern of cross-resistance found with escape mutants (Fig. 3). This was most evident with antibodies H80C25 and AD2 in particular.

Residue 1-221 of HAV aligns with the base of the "FMDV loop" (G-H loop) of VP1 in MV (29). This loop represents an important antigenic site in the aphthoviruses and is known to function as a continuous antigenic determinant in foot-and-mouth disease virus (FMDV) (1, 5). In HAV, the data suggest that this loop is antigenic but potentially independent of the dominant antigenic site described above. Mutations at residue 1-221 were selected only by antibody H7C27, and this antibody neutralized all other escape mutants. In previous collaborative work with Cox and Feinstone, however, we showed that a genetically engineered HM175 variant having a Ser-to-Glu substitution at residue 1-114 was partially resistant to H7C27 but well neutralized by other antibodies (11). Thus, the resistance phenotype of this engineered mutant was similar to that of the 1-221 mutants shown in Fig. 4, suggesting that residues 1-114 and 1-221 may contribute to the same site. However, residue 1-114 of HAV aligns with the base of the B-C loop of VP1 in MV, opposite the residue aligning with 1-102 of HAV. It is thus tempting to speculate that this site is in fact in very close proximity and perhaps part of the immunodominant antigenic site of HAV, but further study will be required to confirm or refute this hypothesis.

Antibodies K34C8 and B5B3 both recognize epitopes within the dominant antigenic site. Although no one mutant was resistant to both K34C8 and B5B3, resistance to several other antibodies (2D2, 3E1, and H80C25) could be conferred by mutations at both residues (3-070 and 1-102) implicated in K34C8 and B5B3 resistance, respectively (Fig. 3). These results are consistent with the fact that the two antibodies bind semiindependently to the capsid surface. They do not compete with each other in solid-phase binding assays but rather demonstrate a binding enhancement effect, suggesting that they recognize closely spaced epitopes (Fig. 2) (31, 34).

Thus, the immunodominant antigenic site must span an area with a diameter at least twice that of a typical Fab fragment footprint.

In crystallographic studies of an antigen-antibody complex, the footprint of an Fab antibody fragment binding to lysozyme has been shown to cover approximately 750 Å^2 of the solvent-accessible surface of the enzyme (2). The complementarity-determining regions of the antibody were found to be in close contact with 16 amino acid residues of the antigen. Similar X-ray crystallographic analysis of complexes of Fab fragments with influenza virus neuraminidase tetramers demonstrated a comparable number of amino acid residues contacted in the antigen (10). It thus seems reasonable to assume that antibodies which neutralize HAV closely contact an approximately equal number of residues within the solvent-accessible surface of the capsid proteins. However, when we examined multiple, independent escape mutants selected for neutralization resistance to individual monoclonal antibodies, we found mutations at only one or, at most, two amino acid positions (Table 2). For example, six of seven mutants selected against 2D2 had mutations at residue 3-070, while all four independently selected H7C27 mutants had amino acid substitutions (due to different nucleotide base substitutions) at residue 1-221. The very restricted number of residues we identified as sites of mutation could reflect very stringent structural constraints imposed by the need to retain biological activity of the capsid. However, these data are also compatible with the existence of key amino acid residues which play a critical role in determining the avidity of particular neutralizing antibodies for the virus capsid.

In interpreting the results of these experiments, we have considered it likely that the sites of mutations which we have identified are located within the region of the capsid which is actually contacted by neutralizing antibody. This is a reasonable assumption, given that this has been shown to be the case for almost all mutations responsible for neutralization escape in those picornaviruses for which the three-dimensional structure has been determined (1, 28, 32). However, a clear-cut exception to this paradigm was recently demonstrated by Parry et al. (30), who found that neutralization resistance could be conferred by amino acid substitutions occurring outside a neutralization epitope on the surface of FMDV (serotype O). Thus, the potential for functionally significant perturbations of the capsid structure occurring at some distance from the site of a mutation must be considered, although such a mechanism of neutralization escape cannot be documented in the absence of definitive crystallographic studies.

Although the work described in this report was carried out entirely with murine monoclonal antibodies, we have recently found that human monoclonal antibodies recognize similar epitopes on the capsid surface. Although these studies remain in progress, partial or complete neutralization resistance to two of three human monoclonal antibodies is conferred by amino acid substitutions at Asp 3-070 or Ser 1-102 (13a). Thus, there is at present little evidence to suggest significant differences in the antibody repertoires developed against HAV in mice and in humans. This contrasts with a previous report that human and murine antibodies differ with respect to their binding sites on the poliovirus capsid (36) and may reflect a relatively limited number of potentially antigenic sites present on the HAV capsid.

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ADDENDUM IN PROOF

Y. Moritsugu and coworkers have isolated neutralization escape variants from the KRM003 strain of HAV (Moritsugu et al., unpublished data). With several exceptions, the locations of escape mutations in the capsid proteins of the KRM003 mutants are very similar to those which we identified in our HM175 escape mutants. However, these workers were not able to establish a pattern of cross-resistance between mutants with substitutions in the vicinity of 3-070 and those with substitutions at 1-100 or 1-174 with the monoclonal antibodies available to them. The KRM003 and HM175 viruses, although both of human origin, are divergent genetically and represent distinctly different genotypes of human HAV (17).

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