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# Neutralization Escape Mutants Define a Dominant Immunogenic Neutralization Site on Hepatitis A Virus

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**Hepatitis A virus is an hepatotrophic human picornavirus which demonstrates little antigenic variability. To topologically map immunogenic sites on hepatitis A virus which elicit neutralizing antibodies, eight neutralizing monoclonal antibodies were evaluated in competition immunoassays employing radiolabeled monoclonal antibodies and HM-175 virus. Whereas two antibodies (K3-4C8 and K3-2F2) bound to intimately overlapping epitopes, the epitope bound by a third antibody (B5-B3) was distinctly different as evidenced by a lack of competition between antibodies for binding to the virus. The other five antibodies variably blocked the binding of both K3-4C8–K3-2F2 and B5-B3, suggesting that these epitopes are closely spaced and perhaps part of a single neutralization immunogenic site. Several combinations of monoclonal antibodies blocked the binding of polyclonal human convalescent antibody by greater than 96%, indicating that the neutralization epitopes bound by these antibodies are immunodominant in humans. Spontaneously arising HM-175 mutants were selected for resistance to monoclonal antibody-mediated neutralization. Fourteen clonally isolated mutants demonstrated substantial resistance to multiple monoclonal antibodies, including K3-4C8–K3-2F2 and B5-B3. In addition, 13 mutants demonstrated a 10-fold or greater reduction in neutralization mediated by polyclonal human antibody. Neutralization resistance was associated with reduced antibody binding. These results suggest that hepatitis A virus may differ from poliovirus in possessing a single, dominant neutralization immunogenic site and therefore may be a better candidate for synthetic peptide or antiidiotype vaccine development.**

Hepatitis A virus (HAV) accounts for 38% of cases of viral hepatitis reported annually in the United States (6). On the basis of morphologic and biophysical characteristics, HAV has been classified among the *Picornaviridae* (5). Like poliovirus, the virion is a naked icosahedron. The capsid contains at least three major structural proteins (VP1, VP2, VP3), of which VP1 appears to be the dominant surface protein (2). Only a single antigenic specificity has been associated with HAV, and significant antigenic variation has not been recognized among different HAV strains (14). Denaturation of the virus leads to a loss of antigenicity, suggesting that the relevant antigenic sites are strictly conformationally dependent (10; J. T. Stapleton, and S. M. Lemon, unpublished data). By covalently cross-linking monoclonal antibodies to intact HAV, one major antigenic site has been located on VP1 (10).

Analysis of mutants that are resistant to neutralizing monoclonal antibodies has proven useful for identifying immunogenic sites involved in the neutralization of other picornaviruses (3, 20, 24). Genomic nucleotide substitutions associated with neutralization resistance, when analyzed in the context of the three-dimensional structure of poliovirus type 1 (PV1) or human rhinovirus type 14 (HRV14), have identified multiple discrete sites on the virion surface which appear to function both as immunogens and as attachment sites for neutralizing monoclonal antibodies (8, 22). Four such neutralization immunogenic sites have been identified on both PV1 and HRV14; they involve all three surface polypeptides (8, 23). In general, mutation occurring within one neutralization immunogenic site has conferred resistance to all monoclonal antibodies binding at that site, but not to those binding at alternate sites.

To identify the neutralization immunogenic sites of HAV, we selected neutalization escape mutants that resist neutralization mediated by a panel of murine monoclonal antibodies. Unlike PV1 and HRV14, characterization of these antibodies and related neutralization escape mutants suggests the existence of only a single neutralization immunogenic site on the HAV virion. Furthermore, this immunogenic site is dominant in human antibody responses to natural infection.

## MATERIALS AND METHODS

**Cells.** Continuous African green monkey kidney (BS-C-1) cells (passages 58 to 87) were cultivated as described previously (25).

**Virus.** The cell culture-adapted HM-175 strain of HAV had previously been passaged 10 times in African green monkey kidney cells and 5 times in Bs-C-1 cells (1). A stock virus seed was prepared from tissue culture supernatant fluids and extracted twice with equal volumes of chloroform (25). The infectivity titer of this HM-175 seed was approximately $10^6$ radioimmunofocus-forming units per ml (15). For immunoassays, virus was concentrated from cell culture supernatant fluids and purified by isopycnic banding in cesium chloride (18).

**Antibodies.** Murine monoclonal antibodies raised against HAV were kindly provided by three other laboratories. Ascitic fluids containing monoclonal antibodies K3-4C8, K2-4F2, and K3-2F2 were obtained from A. G. Coulepis and I. D. Gust, Melbourne, Australia (19); B5-B3 was the gift of R. S. Tedder, London, United Kingdom; and 6A5, 1B9, 2D2, and 3E1 were provided as concentrated hybridoma tissue culture supernatant fluids by J. V. Hughes, E. A. Emini, and R. Gerety, West Point, Pa. (10). Characteristics

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TABLE 1. Murine monoclonal anti-HAV antibodies

| Antibody[a] | Isotype | Immunizing strain | Neutralization titer[b] | Source[c] |
|---|---|---|---|---|
| K3-4C8 | IgG2a | HM-790 | >5.0 | A |
| K2-4F2 | IgG2a | HM-790 | >5.0 | A |
| K3-2F2 | IgG2a | HM-790 | >5.0 | A |
| B5-B3 | IgG2a | KMW-1 | 5.1 | B |
| 6A5 | IgG2a | CR-326 | 6.0 | C |
| 1B9 | IgG1 | CR-326 | 3.5 | C |
| 2D2 | IgG2a | CR-326 | 5.4 | C |
| 3E1 | IgG2a | CR-326 | 4.0 | C |

[a] 6A5, 1B9, 2D2, and 3E1 were supplied as concentrated hybridoma tissue culture fluids, whereas other antibodies were ascitic fluids.

[b] $Log_{10}$ of the antibody dilution effecting 50% neutralization of HM175 virus in the radioimmunofocus inhibition assay (15).

[c] A, A. G. Coulepis and I. D. Gust, Fairfield Hospital, Melbourne, Australia (19); B, R. S. Tedder, Middlesex Hospital, London, United Kingdom, C, J. V. Hughes, E. A. Emini, and R. Gerety, Merck Sharp and Dohme Research Laboratories, West Point, Pa. (10).

of these monoclonal antibodies are shown in Table 1. Polyclonal human sera were obtained from patients convalescing from HAV infection acquired in Africa (JC-pcAb) or Alaska (Sm-pcAb). Commercial immune serum globulin (lot no. NF0035-2) was purchased from Cutter Laboratories, Berkley, Calif. Immunoglobulin G (IgG) was purified from human sera by ammonium sulfate precipitation followed by passage through DEAE-Sephacel (Pharmacia Fine Chemicals, Piscataway, N.J.) (16). Murine ascitic fluid IgG fractions were obtained by ammonium sulfate precipitation. Radioiodination of individual IgG fractions was carried out by a modification of the chloramine T method (16). Monoclonal antibodies were quantitated by a solid-phase, isotype-specific, enzyme-linked immunosorbent assay.

**Solid-phase monoclonal antibody competition assay.** The ability of individual monoclonal antibodies to competitively block the binding of other monoclonal and polyclonal antibodies was determined in competitive radioimmunoassays. Briefly, 100 μl of an anti-HAV positive serum (JC-pcAb), diluted 1:1,000 in 50 mM sodium carbonate buffer pH 9.6, was applied to wells of a polyvinyl chloride microtiter plate (Dynatech Laboratories, Inc., Alexandria, Va.) for 4 h at 37°C. After two washes with phosphate-buffered saline (PBS) containing 0.05% Tween 80, 50 μl of a suspension containing gradient-purified HM-175 HAV was applied and incubated overnight at 4°C. After two further washes with PBS–Tween 80, 25-μl samples of fivefold dilutions of each antibody (1:5 to 1:3125) in PBS were applied to two replicate wells and allowed to incubate for 15 min before the addition of 25 μl of a solution containing 200,000 cpm of $^{125}I$-labeled monoclonal or polyclonal IgG diluted in PBS containing 10% fetal bovine serum. After 4 h of incubation at 4°C, the wells were washed extensively and cut out, and residual bound $^{125}I$ was determined with an LKB Rackgamma gamma counter (LKB Instruments, Turku, Finland).

**In vitro neutralization assay.** Quantitation of infectious HAV was carried out by a radioimmunofocus assay (15). This technique is similar to standard viral plaque assays, except that foci of viral replication (radioimmunofoci) are detected by autoradiography after the removal of the agarose overlay, acetone fixation of the cell sheet, and staining with $^{125}I$-labeled polyclonal antibody (Sm-pcAb). Neutralizing anti-HAV antibody was detected by inhibition of radioimmunofocus formation (13) and was considered present if incubation of a sample with virus resulted in a 50% or greater reduction in the number of observed radioimmunofoci.

**Clonal isolation of HAV.** Clonal mutants of HAV were isolated from radioimmunofocus assay cultures as described previously (17), but with minor modifications. After the period of viral growth, the orientation of the agarose overlay within individual petri dishes was marked. The intact overlay was peeled from the cell sheet and placed inverted at 4°C until the completion of the radioimmunofocus assay and the development of the autoradiograms. The inverted overlay was then carefully realigned over the autoradiogram to its original position, and agarose plugs overlying individual radioimmunofoci were removed with a Pasteur pipette. The agarose plug was suspended in 0.75 ml of Hanks balanced salt solution containing 5% fetal bovine serum, freeze-thawed once, and extracted with an equal volume of chloroform. The resulting HAV suspension was subjected to two additional such cycles of plaque purification, and virus was amplified in 24-well tissue culture clusters (Costar, Cambridge, Mass.) containing BS-C-1 cell monolayers. Agarose harvested from multiple regions not overlying radioimmunofoci did not yield virus.

**HAV neutralization escape mutants.** Chloroform-extracted, cell culture-adapted HM-175 HAV was mixed with an equal volume of monoclonal or polyclonal antibody diluted to $10^{-4.5}$ in Hanks balanced salt solution containing 5% fetal bovine serum and incubated for 1 h at 37°C. Longer incubation periods did not lead to enhancement of viral neutralization. Nonneutralized virus was amplified in BS-C-1 cell cultures maintained in medium containing the related antibody at the same concentration employed in the neutralization reaction. Medium containing antibody was replaced twice weekly, and supernatant culture fluids were assayed for HAV by cDNA-RNA slot-blot hybridization (11). Supernatant fluids had detectable HAV 20 days postinoculation. This supernatant fluid was chloroform extracted and subjected to a second neutralization reaction followed by amplification in the presence of higher concentrations of antibody ($10^{-3.5}$). HAV was detectable by hybridization in supernatant fluids 17 days after inoculation of cultures with this virus. Cells were refed with antibody-free medium, and virus subsequently released into supernatant fluids was chloroform extracted and assayed for susceptibility to neutralization with a $10^{-2}$ dilution of the related antibody. After this neutralization reaction, neutralization-resistant mutants were clonally isolated from radioimmunofocus assay cultures as described above. Altogether, 29 mutants underwent two additional neutralization and clonal selection cycles and were amplified in BS-C-1 cells for further characterization. HAV released into the supernatant fluids of these cultures was chloroform extracted before further study.

**cDNA-RNA hybridization.** An HAV cDNA probe was prepared as described previously (11) from *E. coli* cultures containing the recombinant plasmid $pHAV_{LB}1307$, which has an insert of cDNA complementary to HM-175 virion RNA (26). *E. coli* cultures were the gift of J. Ticehurst of the National Institute of Allergy and Infectious Diseases, Bethesda, Md. (26). Direct blotting (11) of HAV suspensions onto nitrocellulose paper was accomplished with the Minifold-II slot-blot apparatus (Schleicher & Schuell Co., Keene, N.H.). Virus samples were diluted into 0.55 ml of blotting buffer (4.61 M formaldehyde in 7.5× SSC, 1× SSC consists of 0.15 M NaCl–0.015 M sodium citrate) and applied directly to the nitrocellulose paper followed by a single wash with 10× SSC. The nitrocellulose was dried at 23°C for 30 min and then baked at 80°C for 2 to 6 h in a vacuum oven. Prehybridization was carried out for at least 3 h at 42°C with denatured calf thymus DNA (0.1 mg/ml) in prehybridization

buffer (50% formamide, 2.5× Denhardt solution, 5× SSC, 0.1% sodium dodecyl sulfate). $^{32}$P-labeled HAV cDNA insert probes were boiled for 3 min and added to hybridization buffer (identical to prehybridization buffer except for 1× Denhardt solution) to a final concentration of $1 \times 10^6$ to $1.5 \times 10^6$ cpm/ml. Hybridization was carried out at 42°C for 22 to 48 h in sealed plastic bags. Nitrocellulose papers were washed twice in 2× SSC–0.1% sodium dodecyl sulfate at room temperature, followed by two washes in 0.1× SSC–0.1% sodium dodecyl sulfate at 52°C. Annealed cDNA was detected autoradiographically after exposure to X-AR5 film (Eastman Kodak Co., Rochester, N.Y.) for 16 h at −70°C with X-Omat intensifying screens (Kodak).

**Immunoaffinity hybridization.** The ability of monoclonal and polyclonal antibodies to bind to selected neutralization-resistant HAV mutants was assessed in a modified immunoaffinity hybridization assay (11). Wells of a polyvinyl microtiter plate were coated with 100 μl of individual antibodies diluted 1:500 in 50 mM sodium carbonate buffer (pH 9.6) for 4 h at 37°C. A 100-μl sample of 1% bovine serum albumin was added to each well during the last hour of this incubation to prevent nonspecific binding of virus in subsequent steps. After aspiration of the wells and five washes with PBS–Tween 80, 100 μl samples of tissue culture supernatant fluids were added to individual wells and incubated for 16 h at 4°C. The contents of each well (representing virus not bound to the antibody) were aspirated and blotted directly onto nitrocellulose paper. The microtiter wells were then washed five times with PBS–Tween 80, and 100 μl of 0.1 N HCl was added for 30 min at room temperature to elute virus bound to the wells. The contents of each well (now representing virus which had been bound to antibody) were subsequently blotted to nitrocellulose as above. Parental virus (normal HM-175 HAV) was included as a positive binding control, whereas a murine monoclonal antibody not directed against HAV was employed as a negative binding control. Blots were subjected to cDNA-RNA hybridization as described above.

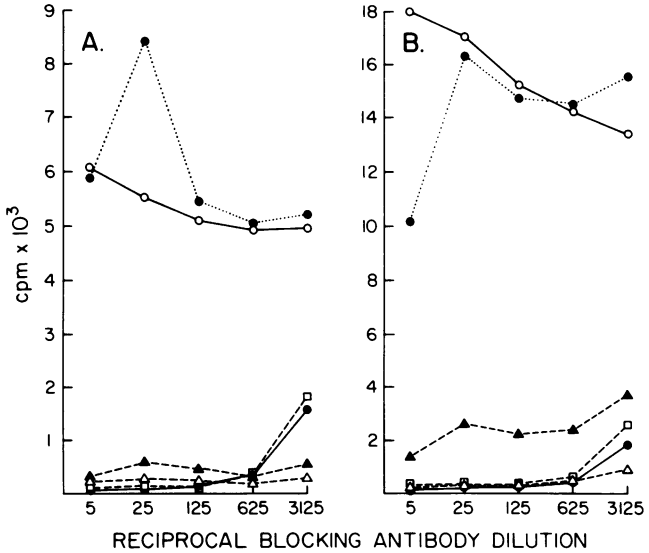


FIG. 1. Competitive inhibition of K3-4C8 and K2-4F2 monoclonal antibody binding to HM-175 virus in solid-phase radioimmunoassays. Various monoclonal antibodies (– – – and · · ·) and polyclonal sera (——) were tested for their ability to inhibit the binding of $^{125}$I-labeled K3-4C8 (A) or K2-4F2 (B) to HM-175 virus as described in the text. Symbols: (○) preimmune chimpanzee sera, (●) JC-pcAb, (●) B5-B3 (□) K2-4F2 (△) K3-4C8, (▲) K3-2F2. Each data point represents the mean of two replicate assay determinations. Results were reproducible.

TABLE 2. Competitive inhibition of monoclonal antibody binding to HM-175 virus in solid-phase radioimmunoassays

| Competing antibody | Inhibition of radiolabeled antibody[a] | | | |
|---|---|---|---|---|
| | K3-4C8 | K3-2F2 | K2-4F2 | B5-B3 |
| K3-4C8 | + + + + | + + + + | + + + + | 0 |
| K3-2F2 | + + + + | + + + + | + + + + | 0 |
| 6A5 | + + + + | + + + + | + + + + | + + |
| K2-4F2 | + + + + | + + + + | + + + + | + + |
| 1B9 | + + + + | + + + + | + + + + | + + + |
| 2D2 | + + + + | + + + + | + + + + | + + + |
| 3E1 | + + + + | + + + + | + + + | + + + + |
| B5-B3 | 0 | 0 | 0 | + + + + |

[a] Results shown are with dilutions of competing antibodies ranging from 16 to 50 μg/ml. 0, <20% reduction in counts per minute with the addition of competing antibody compared with preimmune sera; +, 20 to <40% reduction; + +, 40 to <60% reduction; + + +, 60 to <80% reduction; + + + +, 80 to 100% reduction. Results shown represent the mean counts per minute obtained in replicate microtiter wells in immunoassays and were confirmed in repeat experiments.

## RESULTS

**Topologic mapping of HAV neutralization epitopes.** Each of the eight monoclonal antibody preparations possessed high-titered neutralizing activity against HM-175 strain HAV when assayed in a radioimmunofocus inhibition assay (13), although all were derived from mice immunized with other strains of virus (Table 1).

To characterize epitopes recognized by these monoclonal antibodies, competitive radioimmunoassays were established employing individual radiolabeled monoclonal antibodies. Figure 1 depicts representative preliminary experiments in which multiple dilutions of several different antibodies were tested for their ability to compete for binding with radiolabeled K2-4F2 or K3-4C8. These and similar experiments indicated that K2-4F2, K3-4C8, and K3-2F2 were directed against closely overlapping neutralization epitopes, since these three monoclonal antibodies competed with each other for binding to the virus to a very high degree. In contrast, B5-B3 did not block the binding of radiolabeled K2-4F2 to HM-175 (Fig. 1A) and thus appeared to recognize a distinct epitope. High concentrations of B5-B3 resulted in a moderate reduction of K3-4C8 binding, however (Fig. 1B), whereas some concentrations of B5-B3 caused a reproducible enhancement of K2-4F2 binding (Fig. 1A). These results suggested that these epitopes are closely spaced. Additional data obtained with the complete panel of antibodies are summarized in Table 2. The data in Table 2 are derived from experiments in which the concentration of the unlabeled antibody ranged from 16 to 50 μg/ml and was in considerable excess over the concentration of the labeled monoclonal antibody (<0.5 μg/ml). Monoclonal antibodies 6A5, K2-4F2, 1B9, 2D2, and 3E1 all showed varied abilities to compete for binding with radiolabeled K3-4C8, K3-2F2, K2-4F2, and B5-B3, further suggesting that the epitopes recognized by each of these monoclonal antibodies are closely spaced on the capsid surface and part of a single functional antigenic domain.

**Immunodominance of neutralization epitopes.** Most of the monoclonal antibodies were found to compete effectively, but not completely, with radiolabeled human polyclonal antibody for binding to HM-175 HAV in solid-phase radioim-

TABLE 3. Competitive inhibition of polyclonal human convalescent antibody binding to HM-175 virus by monoclonal antibodies, alone or in combination

| Competing antibodies[a] | Inhibition of radiolabeled convalescent human antibody[b] | |
|---|---|---|
| | JC-pcAb | Sm-pcAB |
| None (PBS) | 4,304–5,900 (0%) | 4,542–3,588 (0) |
| 1B9 | 3,090–3,022 (40) | ND |
| K3-4C8 | 2,674–2,739 (47) | 2,073–1,950 (51) |
| B5-B3 | 2,118–1,962 (60) | 1,150–1,118 (72) |
| 6A5 | 2,292–1,641 (62) | ND |
| 3E1 | 1,834–1,735 (65) | ND |
| K3-4C8–K2-4F2 | 1,629–1,672 (68) | 924–804 (79) |
| 2D2 | 1,577–1,377 (71) | ND |
| K2-4F2 | 1,242–1,210 (76) | 1,225–1,365 (69) |
| 6A5-3E1 | 1,191–1,216 (76) | ND |
| K3-4C8–B5-B3 | 189–190 (96) | 92–136 (97) |
| 6A5–B5-B3 | 142–112 (99) | ND |
| K2-4F2–B5-B3 | 76–52 (99) | 55–49 (99) |

[a] Competing antibodies were present at dilutions yielding maximum inhibition in single antibody competition immunoassays.

[b] Results are given as the range of counts per minute in replicate microtiter plate wells, with the percent reduction from PBS control wells given within parentheses. ND, Not done.

munoassays (Table 3). B5-B3 and K2-4F2 were roughly equivalent in their ability to compete with radiolabeled polyclonal Sm-pcAb (Fig. 2) or JC-pcAb (antibodies obtained during convalescence from two naturally infected humans), but were additive in their inhibitory effects. A 1:1 mixture of B5-B3 and K2-4F2 was equivalent to JC-pcAb in blocking the binding of either Sm-pcAb or JC-pcAb to the virus, indicating that a mixture of these two antibodies is able to block the binding of greater than 99% of the antibodies elicited during natural human infections (Table 3). Other combinations of monoclonal antibodies demonstrated variable degrees of additive effects in inhibiting the binding of radiolabeled polyclonal antibodies to the virus (Table 3). Together, the data presented in Tables 2 and 3 suggest that the neutralization epitopes recognized by these eight monoclonal antibodies form a closely spaced, immunodominant cluster on the capsid surface, although alternative interpretations (i.e., inhibition of antibody binding due to induced changes in capsid conformation) are possible and are considered below (see Discussion).

**Neutralization escape mutants of HAV.** A multistep selection process was employed to select virus resistant to neutralization with K2-4F2, K3-4C8, B5-B3, and polyclonal human serum (JC-pcAb). HM-175 HAV was incubated with a dilution of monoclonal or polyclonal antibody that was approximately 10-fold that required to effect 50% neutralization (endpoint neutralization titer), and surviving virus was propagated in the presence of the same concentration of antibody. Approximately 99% of first-passage HAV survivors selected in this fashion were resistant to a concentration of each related antibody that was 100-fold the endpoint neutralization titer (higher concentrations of antibody were not tested) (data not shown). This supernatant virus was chloroform extracted, and the selection cycle was repeated under pressure of 10-fold higher concentrations of antibody. Second-passage virus released into supernatant culture fluids in the absence of antibody was resistant to a concentration of antibody that was 300-fold the endpoint neutralization titer of that antibody when tested against normal HM-175 virus (data not shown). This included virus selected in the presence of polyclonal antibody. Second-passage mutant virus was subjected to a high-stringency neutralization reaction ($10^{-2}$ dilution of antibody) and plaque purified through three cycles as described in Materials and Methods (the antibody concentration was 3,000-fold the endpoint neutralization titer). Twenty-nine neutralization escape mutants were clonally isolated and amplified in BS-C-1 cells in the presence of low antibody concentrations. These mutants resisted neutralization by the homologous antibody (Fig. 3). Because of the nature of the selection process, it is possible that some of these neutralization escape mutants represent sibling clones.

The genetic stability of three different HM-175 mutants was assessed during a single passage in 890-$cm^2$ roller bottle cultures of BS-C-1 cells, both in the presence and absence of the relevant antibody ($10^{-3}$ dilution). Virus released into the cell culture supernatant fluid 32 days after inoculation was tested for neutralization resistance. In each case, less than a threefold reduction in infectious titer followed incubation of the virus with a $10^{-2}$ concentration of antibody, confirming the genetic stability of the mutations. There was no difference between the neutralization resistance of virus propagated in the presence or absence of the related antibodies.

**Antibody binding to neutralization-resistant HAV mutants.** Resistance to neutralizing monoclonal antibodies could be conferred by mutations altering the ability of antibodies to bind to the viral capsid or by mutations that block the capsid conformational changes that are thought to accompany neutralization of picornaviruses such as poliovirus after the bivalent binding of some monoclonal antibodies (4). To assess these alternative possibilities, neutralization escape mutants were examined for their ability to bind their cognate antibodies. First, mutant 18 (selected against K2-4F2) was amplified in an 890-$cm^2$ roller bottle culture of BS-C-1 cells and purified from 0.1% Sarkosyl cell lysates by isopycnic

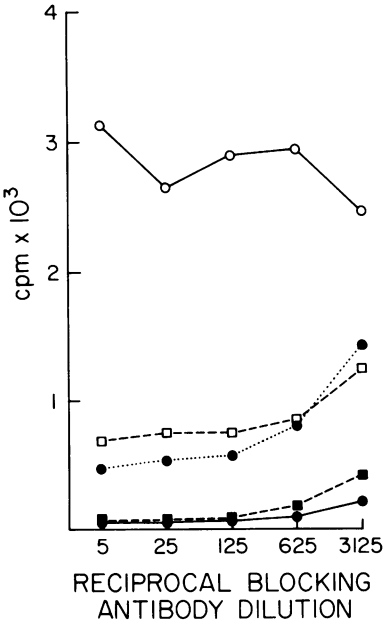


FIG. 2. Two monoclonal antibodies (– – – and · · ·) and polyclonal sera (——) were tested for their ability to inhibit the binding of radiolabeled polyclonal antibody Sm-pcAb to HM-175 virus. Symbols: (○) preimmune chimpanzee serum, (●) B5-B3, (□) K2-4F2, (■) a 1:1 mixture of B5-B3 and K2-4F2, (●) JC-pcAb. See legend to Fig. 1.

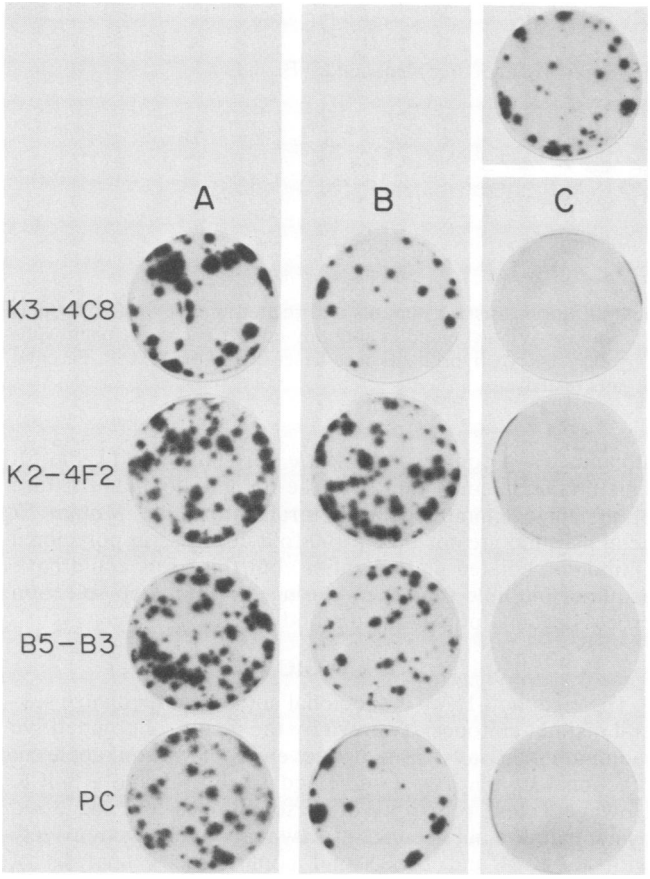


FIG. 3. Clonally isolated, neutralization-resistant HM-175 mutants. Viruses selected for resistance to monoclonal antibodies K3-4C8, K2-4F2, and B5-B3 and the human polyclonal antiserum JC-pcAb were harvested in the absence of antibody, chloroform-extracted, and quantitated by radioimmunofocus assay before (A) and after (B) neutralization with a 1:3,000 dilution of related antibody; thus, foci in (B) represent nonneutralized virus. Autoradiograms in (C) represent cells inoculated with parental HM-175 subjected to identical neutralization reactions. An autoradiogram of cells inoculated with parental virus not reacted with antibody is shown at the top of (C).

banding in cesium chloride. The density gradient profile of mutant 18 was similar to that of the parental HM-175 virus, with the major peak of virus at 1.32 to 1.33 g/cm$^3$ (data not shown). Specifically, there was no increase in the heavy particle fraction banding at 1.40 to 1.44 g/cm$^3$, indicating that the capsid mutation associated with neutralization resistance had not altered the normal impermeability of the capsid to cesium (18). The ability of radiolabeled Sm-pcAb and K2-4F2 antibodies to bind to mutant 18 and parental HM-175 viruses was assessed in solid-phase radioimmunoassays. Microtiter wells were coated with JC-pcAb as described above and incubated overnight at 4°C with gradient fractions containing purified viruses. The wells were washed extensively, radiolabeled Sm-pcAb or K2-4F2 was added, and the assay was completed as described above. The binding of radiolabeled K2-4F2 was markedly decreased, relative to the binding of polyclonal Sm-pcAb, when mutant 18 was compared with the HM-175 control (Table 4). These data suggest that the neutralization resistance of mutant 18 was associated with a substantially reduced affinity of the virus for the monoclonal antibody (K2-4F2) against which it had been selected.

To further assess this hypothesis, the ability of individual monoclonal and polyclonal antibodies to bind to selected mutants was determined by immunoaffinity hybridization. This approach was taken in lieu of radioimmunoprecipitation because of the difficulties inherent in radiolabeling HAV endogenously (due to the lack of inhibition of host cell protein synthesis). Antibody was coated onto the surface of microtiter plate wells. Virus suspensions were incubated in the antibody-coated wells overnight, and unbound virus and virus bound to antibody were assessed by cDNA-RNA hybridization as described in Materials and Methods. Figure 4 depicts the hybridization signals achieved with HAV mutants binding to their cognate antibodies. This was compared with the hybridization signal achieved with virus not bound to antibody after an overnight incubation. Approximately 50% of the parental HM-175 virus included as a control was bound to antibody when slot-blot hybridization results were measured quantitatively by densitometry (data not shown). In contrast, neutralization-resistant mutants were bound to their related antibodies to a much lesser degree, in agreement with the hypothesis that neutralization resistance was conferred by altered affinity of monoclonal antibodies for the immunodominant site on the virus capsid. Selected mutants were also evaluated for binding to three unrelated monoclonal antibodies and the polyclonal antibody JC-pcAb (data not shown). Although largely qualitative, these experiments suggested that the binding of the mutants to most monoclonal antibodies was decreased when compared with parental virus. Additionally, the amount of each mutant binding to polyclonal antibody appeared diminished (albeit to a lesser degree than with monoclonal antibody) when mutants were compared with parental virus. This finding is consistent with the presence of an immunodominant antigenic site.

**Cross-resistance between neutralization-resistant mutants.** Different neutralization immunogenic sites on PV1 and HRV14 have been defined by grouping neutralization escape mutants with resistance among, but not between, various groups of monoclonal antibodies (3, 20, 21, 24). Thus it was of interest to determine whether HAV escape mutants resistant to any one antibody would be broadly resistant to other monoclonal antibodies in the panel. Mutants were characterized for resistance to selected monoclonal and polyclonal antibodies (including immune serum globulin which contains antibody from a large number of human donors) by assessing the reduction in infectious titer after neutralization with a $10^{-2}$ dilution of antibody. Each of the antibodies employed in these experiments possessed a 50% neutralization titer in excess of $10^{-5}$. Approximately $10^5$ RFU of virus was mixed with an equal volume of antibody and incubated for 1 h at 37°C. The mixture was diluted serially in 10-fold increments and assayed by radioim-

TABLE 4. Binding of $^{125}$I-labeled anti-HAV to HM-175 and mutant 18 viruses in solid-phase radioimmunoassays

| $^{125}$I-anti-HAV | Mean cpm bound | | |
|---|---|---|---|
| | HM-175 | Mutant 18(+)[a] | Mutant 18(−)[a] |
| Sm-pcAb | 5,856 | 6,331 | 3,991 |
| K2-4F2 | 5,664 | 708 | 549 |

[a] Mutant 18 ways purified from infected 890-cm$^2$ roller bottle cultures in the presence (+) or absence (−) of K2-4F2 antibody. Results shown represent the mean of two replicate determinations.

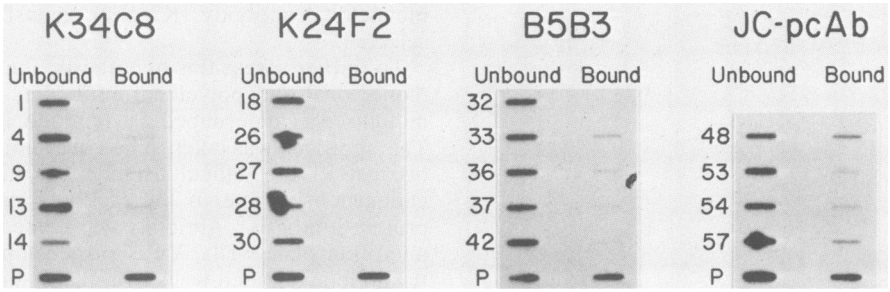


FIG. 4. Immunoaffinity hybridization results obtained with selected neutralization-resistant mutants and related monoclonal antibodies (K3-4C8, K2-4F2, B5-B3) and polyclonal antibodies (JC-pcAb). Virus suspensions were incubated in antibody-coated microtiter plate wells overnight, and unbound virus and virus bound to antibody were assessed by cDNA-RNA hybridization as described in the text. P, Parental HM-175 HAV. Numbers represent the laboratory designations for the mutants tested.

munofocus assay. Although the neutralization resistance of mutants was generally greatest with the cognate monoclonal antibody, extensive cross-resistance among other monoclonal antibodies was exhibited by many mutants (Table 5). Additionally, resistance of selected mutants extended to 6A5, 1B9, 2D2, and 3E1 as well (data not shown). The mutants that demonstrated the highest degree of homologous resistance and that were the most broadly cross-resistant to other monoclonal and polyclonal antibodies were those selected for resistance to K2-4F2. Of the three monoclonal antibodies used in selection of neutralization escape mutants, this antibody had the broadest ability to compete with other monoclonal antibodies in competition immunoassays (Table 2) and was able to effectively block the binding of both K3-4C8 and B5-B3 antibodies to virus. Cross-resistance of escape mutants extended to both B5-B3 and K3-2F2 antibodies, even though there were no apparent interactions between these antibodies in competitive binding assays. These observations strongly support the concept that the epitopes bound by K3-4C8, K2-4F2, and B5-B3 are part of a single functional neutralization immunogenic site.

Many mutants selected for resistance to monoclonal antibodies demonstrated a greater than 10-fold reduction in neutralization by polyclonal JC-pcAb and immune serum globulin when compared with the parental virus (Table 5). This finding is consistent with the immunodominant nature of the major immunogenic neutralization site. Mutant 57 demonstrated greater than a 100-fold decrease in polyclonal antibody-mediated neutralization, but this virus could have acquired multiple mutations during the multistep selection process against polyclonal antibody (Jc-pcAb).

## DISCUSSION

The eight anti-HAV monoclonal antibodies recognize several distinct epitopes as shown by the results of competitive radioimmunoassays. Two distinct epitopes without apparent functional overlap were identified by B5-B3 and K3-4C8. However, close proximity of these epitopes was suggested by competition between K2-4F2 (which overlaps extensively with K3-4C8) and B5-B3 and confirmed by analysis of monoclonal antibodies 6A5, 1B9, 2D2, and 3E1. These latter antibodies recognize epitopes that overlap both B5-B3, K3-2F2, and K3-4C8 to variable degrees (Table 2), suggesting that all of these epitopes are closely clustered on the capsid surface. Several different combinations of monoclonal antibodies that showed minimal competition with each other for binding to the virus (e.g., B5-B3 and K2-4F2) were capable of almost completely blocking the binding of radio-

TABLE 5. Resistance of HM-175 virus mutants to neutralization with monoclonal and polyclonal antibodies[a]

| Mutant | Selection against | Monoclonal antibody | | | | Polyclonal antibody | |
|---|---|---|---|---|---|---|---|
| | | K3-4C8 | K2-4F2 | K3-2F2 | B5-B3 | JC | ISG |
| 1 | K3-4C8 | 0.3 | 0.6 | 0.4 | 0.1 | 0.5 | 1.0 |
| 8 | K3-4C8 | 1.2 | 1.2 | 1.0 | 0.4 | 0.4 | 1.5 |
| 18 | K2-4F2 | 0.5 | −0.2 | 0.0 | 0.1 | 1.2 | 1.4 |
| 19 | K2-4F2 | 0.8 | 0.1 | 0.2 | 0.3 | 1.6 | 2.4 |
| 20 | K2-4F2 | 0.4 | −0.1 | 0.0 | 0.2 | 0.8 | 1.0 |
| 23 | K2-4F2 | 1.1 | 0.3 | 0.7 | 0.6 | 1.5 | 1.6 |
| 27 | K2-4F2 | 0.7 | 0.1 | 0.2 | 0.1 | 1.0 | 1.2 |
| 28 | K2-4F2 | 0.9 | 0.2 | 0.7 | 0.5 | 1.1 | 1.6 |
| 30 | K2-4F2 | 0.2 | 0.4 | 0.4 | 0.6 | 1.7 | 1.5 |
| 32 | B5-B3 | 0.7 | 0.8 | 0.7 | 0.3 | 1.0 | 1.1 |
| 33 | B5-B3 | 0.7 | 0.9 | 0.6 | 0.2 | 1.0 | 1.5 |
| 34 | B5-B3 | 1.1 | 1.1 | 1.1 | 0.4 | 1.3 | 1.3 |
| 36 | B5-B3 | 1.3 | 1.2 | 0.5 | | 1.4 | 2.1 |
| 57 | JC | 0.4 | 0.4 | 0.4 | 0.2 | 0.4 | 0.8 |
| HM-175[b] | | 2.4–2.8 | 2.5–2.6 | 1.6–1.8 | 1.3–2.4 | 2.5–3.2 | 2.9–3.9 |

[a] The data for all mutants but HM-175 represent the greatest $\log_{10}$ reduction in infectious titer of virus after 1 h of incubation with a $10^{-2}$ dilution of antibody in duplicate experiments.

[b] The data for HM-175 represent the range of $\log_{10}$ reductions in titer of parental virus in four separate experiments.

labeled human polyclonal antibodies (Table 3). Thus, the monoclonal antibodies appear to be directed against components of a single functional domain that is immunodominant in naturally infected humans as well as in experimentally immunized mice.

Although the results of these competition experiments are consistent with direct competition between antibodies at their respective epitopes, other possibilities need to be considered. Reduced binding of radiolabeled antibody in competition immunoassays could occur as a result of a capsid conformational change induced by binding of the nonlabeled antibody. However, this interpretation appears unlikely for the following reasons. The variable inhibition of B5-B3 binding by other monoclonal antibodies (Table 2) could be explained only by multiple different capsid conformational states, whereas available evidence derived from studies with poliovirus supports the existence of only two alternate capsid conformations (4). Similarly, the additive effect of several combinations of antibodies in blocking the binding of polyclonal antibody (Table 3) is difficult to explain on the basis of conformational changes, because this would also require the existence of multiple conformational states. These considerations, and the fact that combinations of noncompeting monoclonal antibodies were most likely to show significant additive effects in blocking the binding of polyclonal antibody, suggests competition at the antibody binding site. An additional consideration is that the binding of antibody to any antigenic site on a picornavirus could in theory interfere nonspecifically with the binding of other antibodies due simply to crowding on the virion surface. This, however, was not observed with K3-4C8–K3-2F2 and B5-B3 which did not compete with each other under the conditions employed (Table 2).

The existence of a single immunodominant neutralization site on the HAV capsid was further suggested by analysis of spontaneously occurring HAV mutants that were resistant to monoclonal antibody-mediated neutralization. Mutants were selected by passage of HM-175 virus in the presence of either individual murine monoclonal antibodies or polyclonal human convalescent serum. Neutralization resistance was associated with reduced affinity of antibody for the virus and extended broadly to most monoclonal antibodies tested, including K3-4C8 and B5-B3 (Table 5). Cross-resistance of escape mutants to monoclonal antibodies recognizing different neutralization immunogenic sites has not been observed with PV1 or HRV14 and has served to functionally classify these sites (20, 23). Thus the data shown in Table 5 suggest that the epitopes recognized by K3-4C8, K2-4F2, and B5-B3 are functionally part of a single immunogenic site.

Despite evidence that the involved antigenic domain is immunodominant in humans (Table 3), neutralization escape mutants remained readily detectable in polyclonal antibody-based immunoassays and radioimmunofocus assays. Also, whereas binding of related monoclonal antibodies to the mutants appeared substantially reduced in comparison to parental HM-175 virus, some degree of antibody binding persisted (Table 4). Emini et al. (4) have suggested that neutralization of poliovirus by some monoclonal antibodies requires the bivalent attachment of antibody to the virus and may be associated with substantial conformational shifts of the capsid alluded to above. It is tempting to speculate that neutralization of HAV has a similar basis, and that some mutations conferring neutralization resistance may prevent the bivalent attachment of antibody but still allow monovalent binding of antibody under conditions of reduced affinity. Significantly, virus with mutations resulting in total elimination of antibody binding to the dominant immunogenic site may not have been identified under the conditions we employed, given the apparent dominance of this antigenic site and the fact that foci of viral replication are recognized immunologically in the radioimmunofocus assay.

Neutralization resistance associated with decreased binding of antibody to virus may result from mutations in the genomic region encoding the antibody binding site (12, 23). Alternately, neutralization resistance could result from mutations distant from the binding site, causing either local perturbations in the antigenic site or a major conformational alteration of the capsid with potential inhibition of a large range of antibodies directed against many sites on the capsid (3). Studies with other picornaviruses (HRV14 and PV1), when placed in the context of the three-dimensional structure of the virion determined by X-ray crystallography, suggest that the site of mutation does in fact reside within or near the monoclonal antibody binding site (20, 23). A single amino acid substitution has been identified in the hemagglutinin molecule of some influenza virus escape mutants, and crystallographic studies have confirmed that one such mutation induces only a local alteration in the conformational structure of the hemagglutinin molecule (12). Given this evidence, it is likely that RNA sequence analysis of HAV neutralization escape mutants will identify the neutralization epitopes of this virus. Such studies will be required to ultimately confirm the existence of a single immunodominant antigenic site on the virus capsid.

Significant antigenic variation has not been identified among HAV strains (14), and epitopes recognized by the monoclonal antibodies appear to be highly conserved among diverse HAV strains (S. M. Lemon et al., unpublished data). This observation stands in sharp contrast to the frequency with which neutralization escape mutants with altered monoclonal antibody binding characteristics arise in vitro. The frequency with which normal HM-175 strain HAV survives a single neutralization reaction varies from $3 \times 10^{-3}$ for monoclonal antibodies to $1.3 \times 10^{-5}$ for immune serum globluin (J. T. Stapleton, unpublished data), consistent with the transcriptional error rate known to accompany replication of RNA viruses (9). Presumably, neutralization-resistant HAV mutants arise during natural infections but do not replicate to significant levels in vivo. It is intriguing to consider, therefore, that the immunodominant neutralization epitopes of HAV may have a vital functional role in the replication of the virus in vivo. If this is true, then neutralization escape mutants may have lost a function necessary for in vivo replication and may be partially or completely attenuated.

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