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Four Chimpanzee Monoclonal Antibodies Isolated by Phage Display Neutralize Hepatitis A Virus

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Chimpanzee immunoglobulins are virtually identical to human immunoglobulins and may have clinically useful applications. Four chimpanzee monoclonal antibodies (MAbs) to the hepatitis A virus (HAV) capsid were isolated from a combinatorial cDNA library of $\gamma 1/\kappa$ antibody genes using phage display. Competition assays indicated that three of the MAbs recognized the same or overlapping epitopes, whereas the fourth recognized a different, nonoverlapping epitope on the HAV capsid. All four MAbs neutralized the homologous HAV strain, HM-175, in a radioimmunofocus assay and two of the four MAbs neutralized a heterologous simian HAV strain, AGM-27. From these data, we conclude that the MAbs must recognize at least three epitopes on the HAV capsid. Furthermore, competition assays performed with neutralizing murine MAbs suggested that three of the chimpanzee MAbs recognized epitopes on the HAV capsid which have not been defined previously.

Key Words: HAV; neutralization; Fab; phage display; antibody library.

INTRODUCTION

Hepatitis A virus (HAV), a positive-sense RNA virus, is a member of the family Picornaviridae. It is the causative agent of hepatitis A and is transmitted via the fecal–oral route, mainly through contaminated water supplies and food sources. Humoral immunity provides an effective defense against hepatitis A. Prior to the availability of the current inactivated virus vaccines, pooled human immune globulin preparations were routinely used to protect individuals traveling to areas of the world where hepatitis A is endemic (Hollinger and Ticehurst, 1996). Unlike the neutralization of other members of the Picornaviridae, such as poliovirus and human rhinovirus, very little is understood about antibody-mediated neutralization of HAV. This is despite the fact that some strains of the virus grow in cell culture, albeit much less efficiently than do other picornaviruses.

The hepatitis A virion is an icosahedron composed of pentamers of three structural proteins, VP1, VP2, and VP3. A fourth structural protein found in other picornaviruses, VP4, is truncated in HAV and has not been identified in virions. Epitopes recognized by neutralizing antibodies on the HAV capsid are dependent on the conformation of the antigen. Very few neutralizing antibodies have been generated to expressed antigens or subunit proteins. Therefore, the mapping of these epitopes has been achieved by a laborious process of generating antibody-escape mutants by serially passaging the virus in cell culture in the presence of neutralizing mouse MAbs. The majority of the mutations selected thus far appear to form an immunodominant site comprising amino acids from both VP1 and VP3. A relatively small number of mutations accounts for escape of these mutants from neutralization by most of the neutralizing murine MAbs available. For example, HM-175 viruses with single amino acid mutations in either VP3 at position 70 or 74, or VP1 at positions 102, 117, 176, or 221 escaped neutralization by 21 of 22 mouse MAbs tested (Ping and Lemon, 1992).

Bacteriophage particles displaying libraries of antibody fragments on their surface have provided a powerful tool for the generation of human monoclonal antibodies (MAbs) to a variety of infectious agents (e.g., human immunodeficiency virus type 1 (Ditzel *et al.,* 1997), hepatitis C virus (Plaisant *et al.,* 1997), Ebola virus (Maruyama *et al.,* 1999)), as well as to cancer markers (e.g., melanoma (Pereira *et al.,* 1997), adenocarcinoma (Henderikx *et al.,* 1998), and ovarian carcinoma (Figini *et al.,* 1998)). MAbs produced from such human antibody gene libraries have the potential to serve directly as immune prophylactic or therapeutic reagents against infectious agents.

Chimpanzees are susceptible to infection with HAV and can produce antibodies which neutralize the virus. Chimpanzee immunoglobulins are virtually identical to

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	FR1	CDR1	FR2	CDR2
HAV#4	EVQLLEQSGAEVKKPGASKVKSCKASGYRFS	NYAMH	WVRQAPGQSLEWMG	WINPVSGKTQFSQKFQG
HAV#5	••••••••••••••••E•LTI••QG••DIFT	••WIG	••••M••KG•••••	I•Y•RNSD•KY•PS•••
HAV#6	•••••••••PRLV••SQTLSLT•SV••ASLT	RGNYYWS	•M••P••KG•••I•	T•HSRGRPAYNTSLAS
HAV#14	••••••••••••••••E•L•I•••V••S•ST	••WIA	••••M••KG•••••	I•S•SDST•KY•PA•••

	FR3	CDR3	FR4
HAV#4	RVTITRDTSASTVYMEVTSLTSEDTAVYYCTR	DLPGTWNFVDVFDI	WGQGTMVTVSS
HAV#5	L•••SA•K•TN•A•LQWN••GAS•••I•••V•	ASY•NY•YFYNM•V	••R••T••I••
HAV#6	••AMSV•A•NNQFSLNLN•VTAA•••••••A•	VFQSKGGA•WAPTTEWTYSYYYYMDV	••R••T•••••
HAV#14	Q•••SA•K•IN•A•LQWS••RAS•••I•••AK	STI•V•DYYYYM•V	••E••S••I••

FIG. 1. A comparison of the amino acid sequences of the γ1-heavy chains of the four HAV-specific MAbs.

those of humans so they have the same potential as human antibodies for clinical applications. We have used a bone marrow derived cDNA library of antibody genes displayed as Fab fragments on the surface of bacteriophage particles to isolate chimpanzee monoclonal antibodies to the HAV capsid. These MAbs may be useful in further defining the properties of neutralizing antibodies that prevent hepatitis A as well as for clinical applications.

The combinatorial antibody library described in this article was generated from a chimpanzee that had been infected with the five recognized hepatitis-causing viruses, hepatitis A, B, C, D, and E viruses. The chimpanzee was seropositive for antibodies to all five viruses. Previously, we used this antibody library to isolate MAbs to the hepatitis E virus capsid protein (Schofield *et al.,* 2000). In this second analysis of the library, we have used inactivated whole HAV particles as the panning antigen to isolate four MAbs to the HAV capsid. Their characterization, in terms of neutralization activities and epitope topographies, are described.

RESULTS

Isolation of HAV-specific Fabs and sequence analysis

After three rounds of panning on formalin-inactivated HAV particles coated to ELISA wells, 300 clones were analyzed for HAV-specificity by ELISA. Twenty-four clones were determined to be HAV-specific.

The restriction enzyme *Bst*NI cuts frequently in the human 1-heavy chain (Marks *et al.,* 1991). This enzyme was used to screen for different heavy-chain sequences among the Fab clones. Several different digestion patterns were observed (data not shown), suggesting that more than one heavy chain sequence was present.

Sequence analysis was performed on 13 of the 24 clones. From these 13 clones, four unique 1-heavy chains were identified. They were represented by clones HAV#4, HAV#5, HAV#6, and HAV#14 (Fig. 1).

We attempted to determine the specific germ-line origin of the four clones by conducting a sequence similarity search of all the known human immunoglobulin genes. The findings are summarized in Table 1.

Neutralization of HAV strain HM-175

The ability of the four MAbs, as Fab fragments, to neutralize HAV *in vitro* was determined by radioimmunofocus assay (RIFA). The HAV strain used in the neutralization assay, HM-175, was the same as the panning antigen. The virus was mixed individually with each of the four purified Fabs and incubated at room temperature for 2 h, and then serial 10-fold dilutions were inoculated onto confluent 11-1 cell monolayers. The monolayers were overlaid with medium containing agarose and incubated at 34.5°C. After 10 days, the cells were fixed and stained and the foci of infected cells were detected by autoradiography. All four Fabs neutralized the HM-175 strain of HAV (Fig. 2).

Neutralization of the simian HAV strain AGM-27

The AGM-27 strain is one of the most divergent HAV strains identified thus far (Tsarev *et al.,* 1991). In the capsid proteins there are 28 amino acid changes compared to the human strain HM-175 (1 amino acid change in VP4, 6 amino acid changes in VP2, 7 amino acid

TABLE 1

Classification of Chimpanzee γ1-Chains of the Four HAV-Specific MAbs, Based on Nucleotide Sequence Homology with Human Immunoglobulin Germ Line Genes

MAb	V_H family	V_H segment	D segment	J_H segment
HAV#4	VH1	DP-25[a]	D1-7[b]	JH3b
HAV#5	VH5	DP-73[a]	D4-11[b]	JH6c
HAV#6	VH4	DP-78[c]	D1-1[b]	JH6c
HAV#14	VH5	DP-73	ND	JH6c

[a] Tomlinson *et al.,* 1992.
[b] Corbett *et al.,* 1997.
[c] V-BASE database.
ND, not determined due to lack of identifiable homologue.

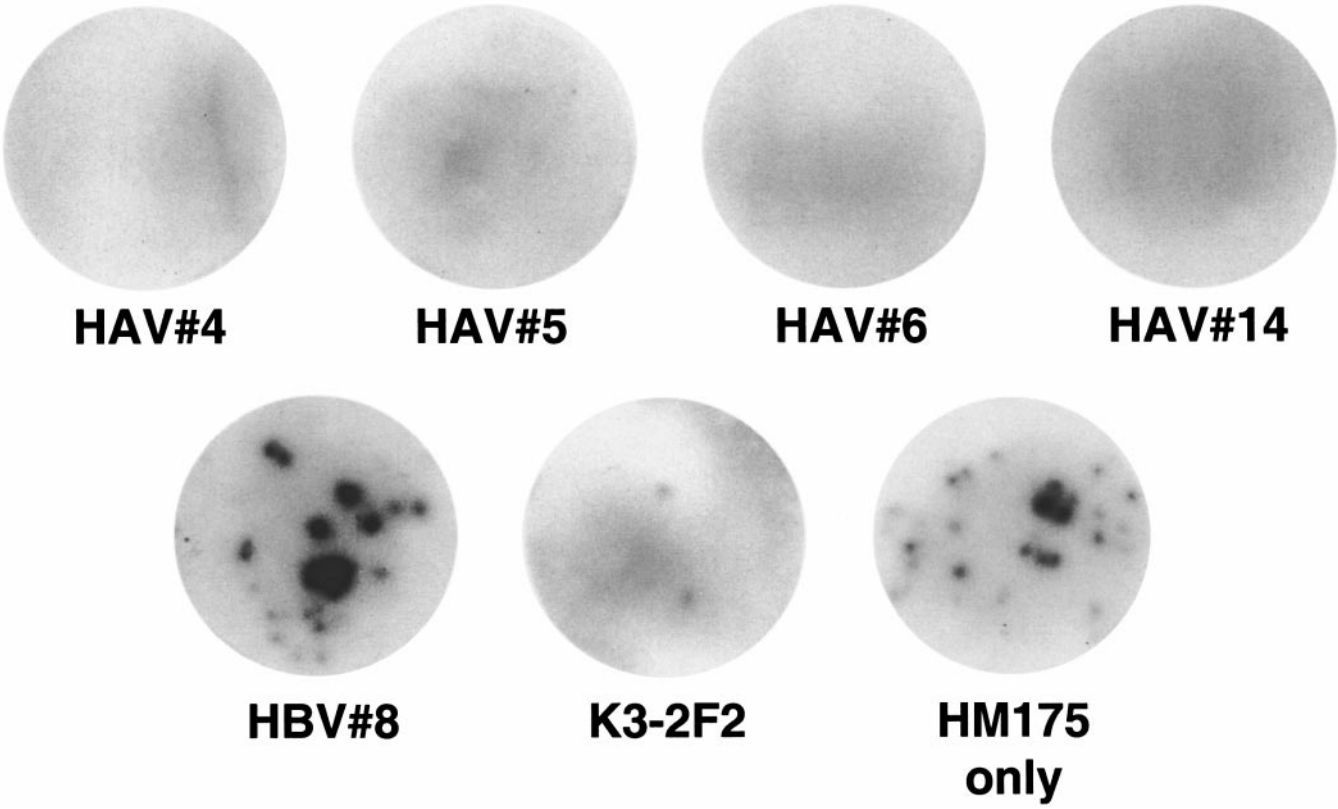

FIG. 2. Assay of neutralization of HAV strain HM-175. Chimpanzee Fabs HAV#4, HAV#5, HAV#6, HAV#14 neutralized the HM-175 strain of HAV, whereas an irrelevant Fab, HBV#8 did not. Murine MAb K3-2F2 is a neutralization-positive control (see text).

changes in VP3, and 14 amino acid changes in VP1). These amino acid changes include one of the two mutations identified in VP3 and two of the four amino acid mutations identified in VP1 as being involved in virus escape from neutralizing mouse MAbs (Ping *et al.,* 1988; Ping and Lemon, 1992; Stapleton and Lemon, 1987).

The ability of the four Fabs to neutralize AGM-27 was determined by RIFA. Each of the four Fabs was incubated with virus as above, and the mixtures were serially diluted prior to inoculation onto 11-1 cells. After 14 days incubation at 34.5°C, the cells were fixed and the RIFA was performed. Examples of the RIFA coverslips are shown in Fig. 3. Four well-characterized mouse MAbs were used as controls. Mouse MAbs K2-4F2, K3-2F2, and B5B3 did not neutralize the AGM-27 strain, whereas mouse MAb K3-4C8 did neutralize the AGM-27 strain as previously reported by Tsarev *et al.* (1991). Of the chimpanzee Fabs, HAV#4 and HAV#6 neutralized the AGM-27 strain but HAV#5 and HAV#14 did not. Furthermore, HAV#4 and HAV#6 bound to viral antigen in 11-1 cells infected with AGM-27 as assayed by immunofluorescence microscopy. However, neither HAV#5 nor HAV#14 bound to antigen in this assay (data not shown).

Neutralization of an HM-175 VP3-070 mutant

Neutralization escape mutants generated in the presence of the mouse MAb K2-4F2 usually result in an amino acid change of $Asp_{70} \rightarrow Ala_{70}$ in the VP3 capsid protein (Nainan *et al.,* 1992; Ping *et al.,* 1988; Ping and Lemon, 1992). This mutation is also present in the VP3 protein of the AGM-27 strain. Since HAV#5 and HAV#14 did not neutralize the AGM-27 strain, we tested for neutralization of an HM-175 variant that had the VP3 $Asp_{70} \rightarrow Ala_{70}$ mutation but otherwise was identical to the HM-175 strain (J. Graff, personal communication). The neutralization assays were performed as described above. Each of the four chimpanzee Fabs neutralized the HM-175 VP3-070 mutant (Fig. 4). The control murine MAb K2-4F2 did not.

Epitope mapping with Fabs

Indirect competition assays were performed to determine whether the four Fabs recognized similar or overlapping epitopes on the HAV capsid (Table 2). Unlabeled HAV#4, HAV#5, and HAV#14 blocked the binding of biotinylated HAV#4, HAV#5, and HAV#14 by >60%. Therefore, these three Fabs recognize the same or overlapping epitopes on the HAV capsid. Unlabeled HAV#6 did not significantly block the binding of the other three biotinylated Fabs and vice versa. Therefore, HAV#6 recognized an epitope on the HAV capsid distinct from that recognized by any of the other three Fabs. Indirect competition assays were also performed with the four Fabs and three well-characterized mouse MAbs (Table 2). The binding of

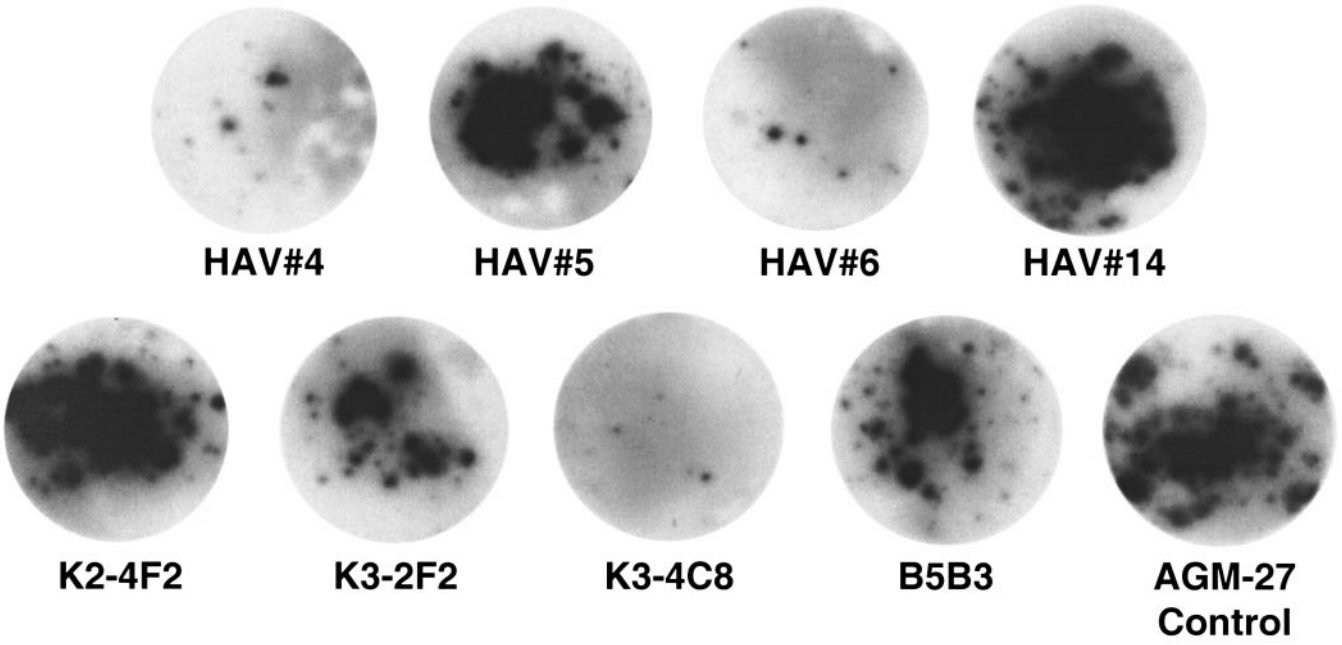

FIG. 3. Assay of neutralization of the simian HAV strain AGM-27. Chimpanzee Fabs HAV#4 and HAV#6 neutralized AGM-27; however, chimpanzee Fabs HAV#5 and HAV#14 did not. Mouse MAb K3-4C8 neutralized AGM-27, mouse MAbs K2-4F2, K3-2F2, and B5B3 did not neutralize AGM-27.

MAbs K3-2F2 and K2-4F2 was inhibited approximately 50–60% by each of the four Fabs. Binding of MAb K3-4C8 to HM-175-coated wells was inhibited ≥84% by HAV#4, HAV#5, and HAV#14. This suggested that these three Fabs and mouse MAb K3-4C8 recognized either the same epitope or an overlapping epitope. HAV#6 inhibited

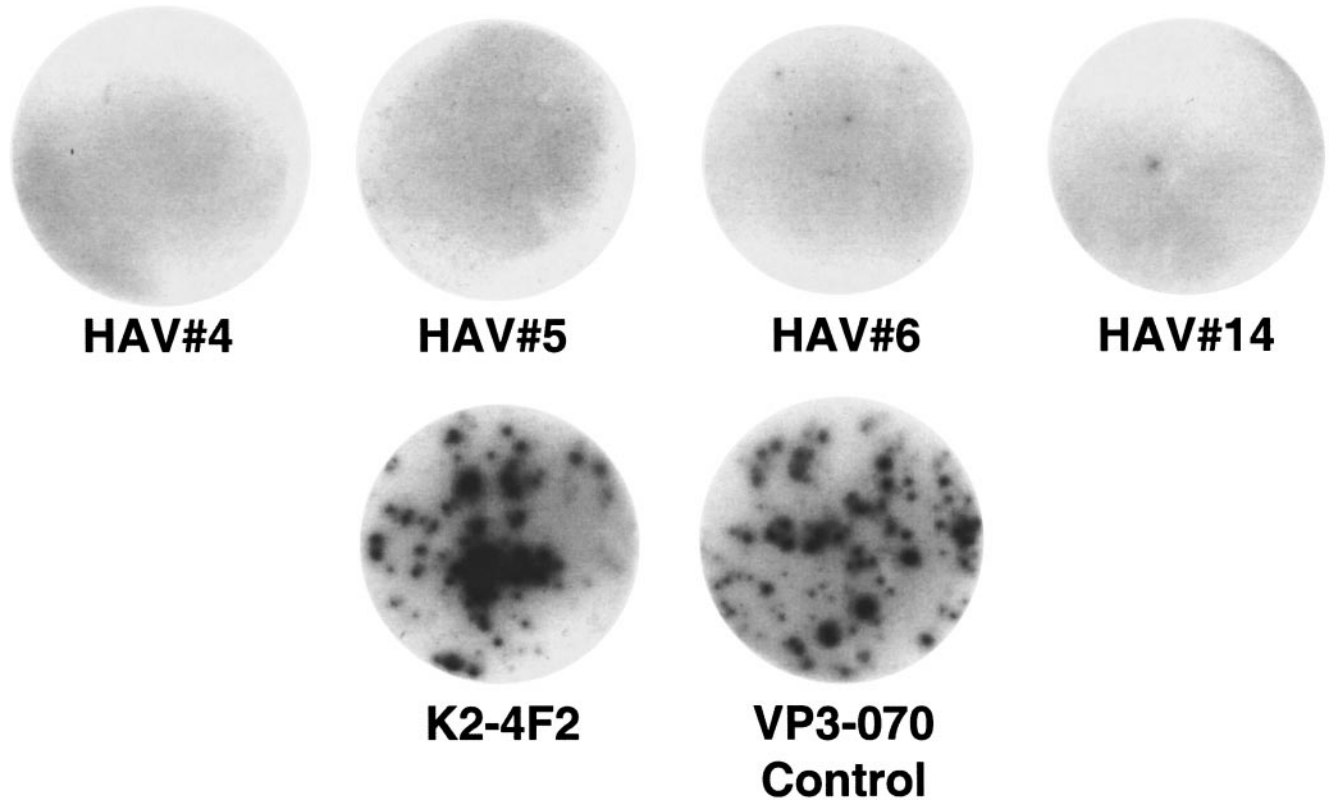

FIG. 4. Assay of neutralization of HAV strain HM-175 carrying the VP3 $Asp_{70} \rightarrow Ala_{70}$ mutation. Chimpanzee Fabs HAV#4, HAV#5, HAV#6, and HAV#14 neutralized this mutant virus. Murine MAb K2-4F2 is a neutralization-negative control (see text).

TABLE 2

Indirect Competition Assay Data Using Fabs HAV#4, HAV#5, HAV#6, and HAV#14 to Block the Binding of Biotinylated Chimpanzee Fabs and Mouse MAbs K2-4F2, K3-2F2, and K3-4C8

	2nd MAb (biotinylated)						
1st MAb	HAV#4	HAV#5	HAV#6	HAV#14	K3-2F2	K3-4C8	K2-4F2
HAV#4	85[a]	86	35	94	50	97	52
HAV#5	87	89	53	94	64	97	63
HAV#6	(6)[b]	28	92	(10)	48	61	48
HAV#14	65	61	22	81	51	84	49

[a] Percentage inhibition of antibody binding to HM-175-coated wells.
[b] Number in parentheses indicates percentage enhancement of antibody binding to HM-175-coated wells.

K3-4C8 binding by 61%, suggesting that the two corresponding epitopes overlap to some extent or are at least spatially close together on the HAV capsid.

The competition assays performed in this study were subject to a number of variables which made quantitative cutoff values for competing and noncompeting antibody pairs very difficult to specify. For example, detection of biotinylated Fab bound to HM-175-coated ELISA wells was much more sensitive than detection of unlabeled antibodies with secondary antispecies antibodies conjugated with alkaline phosphatase. Other factors such as differences in antibody affinities for both primary and secondary antibodies were unknowns which complicated the data further. Therefore, within the context of each assay we described antibodies as "competing" when >60% inhibition of binding was observed, i.e., the two epitopes recognized were likely to be the same or to overlap extensively (Table 2; summarized in Table 3). However, an antibody described as "noncompeting" did not necessarily mean that there was no competition between an antibody pair, rather that there was less extensive overlap between two epitopes.

Epitope mapping with IgGs

Indirect competition assays were also performed between the chimpanzee MAbs as whole IgG molecules and the mouse MAbs K3-2F2, K3-4C8, K2-4F2 (MacGregor *et al.,* 1983), and mouse MAb B5B3 (Biogenesis). Whole IgGs were tested to overcome any differences in antibody affinity that may have arisen from comparing monovalent chimpanzee Fab fragments and bivalent mouse IgG molecules. This protocol would also exclude differences in steric hindrance due to molecular size disparities between Fab and IgG molecules. Here the assay was performed in reverse with the mouse MAbs used to block the binding of the four chimpanzee IgG MAbs. Mouse MAb K3-4C8 reduced the binding of HAV#4, HAV#5, and HAV#14 IgG to around 20–40% of the respective controls. Mouse MAbs K3-2F2, K2-4F2, and B5B3 could only reduce binding of HAV#4, HAV#5, and HAV#14 IgG to a maximum of 60%, compared to the respective controls. An example is shown in Fig. 5. All four mouse MAbs had little effect upon the binding of HAV#6 IgG to HM-175-coated wells. HAV#6 IgG binding

TABLE 3

Summary of Neutralization Assay Data and Epitope Mapping Data for Chimpanzee MAbs HAV#4, HAV#5, HAV#6, and HAV#14, and Mouse MAbs K2-4F2, K3-2F2, K3-4C8, and B5B3

	Neutralization			Epitope mapping[a]			
Antibody	HM-175	VP3-070	AGM-27	HAV#4[b]	HAV#5	HAV#14	HAV#6
HAV#4	Yes	Yes	Yes	Yes	Yes	Yes	No
HAV#5	Yes	Yes	No	Yes	Yes	Yes	No
HAV#14	Yes	Yes	No	Yes	Yes	Yes	No
HAV#6	Yes	Yes	Yes	No	No	No	Yes
K2-4F2	ND[c]	No	No	No	No	No	No
K3-4C8	ND	ND	Yes	Yes	Yes	Yes	No
K3-2F2	Yes	ND	No	No	No	No	No
B5B3	ND	ND	No	No	No	No	No

[a] "Yes" is defined as >60% inhibition of binding to HM-175-coated ELISA wells for each antibody pair.
[b] Competition assays among the four chimpanzee MAbs were performed with Fab fragments; competition assays between the chimpanzee MAbs and the mouse MAbs were performed with whole IgG molecules.
[c] ND, not determined.

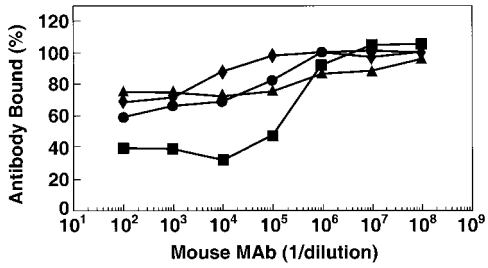

FIG. 5. Indirect competition assay between HAV#4 IgG, and the mouse MAbs, K3-2F2 (▲), K3-4C8 (■), K2-4F2 (●), and B5B3 (◆). The greatest inhibition of HAV#4 IgG binding was with K3-4C8 preincubated with HAV-coated ELISA wells.

was reduced maximally to 60% of the control. These data confirmed those obtained with the Fab fragments.

Cell receptor-blocking assay

Indirect competition assays were performed to determine whether these chimpanzee MAbs could inhibit virus–cell receptor interaction. When HM-175-coated wells were incubated with the Fabs prior to the addition of the soluble HAV cell receptor, HAVCR1 (derived from African green monkey kidney cells (Silberstein *et al.,* 2001)), blocking of receptor binding to the HM-175 was not observed (Fig. 6). In the reciprocal assay, HAVCR1 was unable to inhibit the binding of any of the Fabs to HM-175-coated wells (data not shown). The above was also repeated with the chimpanzee MAbs as whole IgGs. Again inhibition was not observed with either IgG or HAVCR1 as the blocking agent (data not shown).

DISCUSSION

The cDNA library used to isolate the antibodies to HAV is a potential repository for antibodies to all five recognized human hepatitis viruses, HAV, HBV, HCV, HDV, and HEV, since the donor chimpanzee had been experimentally infected with each of the hepatitis viruses. In the first analysis of the library, two neutralizing MAbs directed to the putative HEV capsid protein were identified (Schofield *et al.,* 2000). In this second analysis of the library, four MAbs directed to the HAV capsid were identified. The 1-heavy chains of the HAV MAbs were most closely related, at the nucleotide level, to 1-heavy chains from the human VH1, VH4, and VH5 gene families; such chimpanzee-derived immunoglobulin sequences differ from human-derived sequences no more than genetically distinct human sequences differ from each other. Furthermore, human-derived immunoglobulins exhibit the same half-life in chimpanzees as they do in humans, suggesting that human antibodies are recognized as self and not foreign antigens.

Competition assays suggested the MAbs recognized more than one epitope on the HAV capsid since the three MAbs competed strongly with each other (HAV#4, HAV#5, and HAV#14) for binding but the fourth (HAV#6) competed poorly. All four MAbs neutralized the HAV strain HM-175, the same strain used as the panning antigen. Two of the four MAbs also neutralized the divergent AGM-27 strain and two did not. This, taken with the competition assay data, indicated that there were at least three different epitopes recognized by the four MAbs. Of the three MAbs which competed with each other, HAV#4 recognized a unique epitope since the MAb neutralized both HM-175 and AGM-27, whereas a different epitope was recognized by HAV#5 and HAV#14 since these two MAbs neutralized HM-175 but not AGM-27; the noncompeting MAb, HAV#6, recognized a third epitope (this MAb neutralized both HM-175 and AGM-27).

Competition assays were performed with the four chimpanzee MAbs, as both Fabs and whole IgG molecules, and the mouse MAbs K3-2F2, K3-4C8, K2-4F2, and B5B3. We have attempted to draw a topographical map of the antibody epitopes recognized by the murine and chimpanzee MAbs based on our competition data and other published data (Ping *et al.,* 1988; Ping and Lemon, 1992; Stapleton and Lemon, 1987) (Fig. 7). In accordance with previously published data, the epitopes are closely spaced; the majority of the chimpanzee MAb epitopes are shown closely overlapping with that of the murine MAb K3-4C8. The competition and neutralization data (summarized in Table 3) suggested that MAb K3-4C8 and HAV#4 either recognized the same epitope or two epitopes which overlap extensively. In contrast, the data overall suggested that the other three chimpanzee MAbs (HAV#5, HAV#6, HAV#14) recognize epitopes different from those recognized by the majority of neutralizing murine MAbs. Since escape mutants implicated VP3-070, VP1-102, and VP1-221 as critical for neutralization by the murine MAbs, it can be presumed that these amino acids are not components of the epitopes recognized by the chimpanzee MAbs. Indeed, VP3-070 has been experimentally excluded since all four chimpanzee MAbs neutralized the HM-175 VP3-070 mutant and two MAbs, HAV#4 and HAV#6, neutralized the AGM-27 virus which also has the VP3-070 mutation. The fact that AGM-27

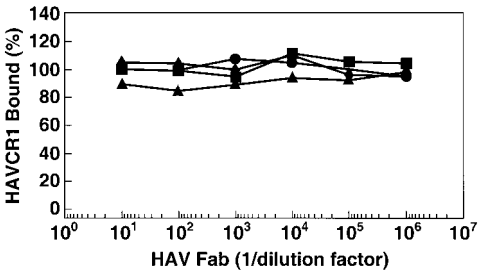

FIG. 6. Virus-cell receptor blocking assay, with a range of dilutions of blocking Fabs HAV#4 (◆), HAV#5 (■), HAV#6 (▲), and HAV#14 (●), and a constant amount of soluble receptor HAVCR1. None of the four Fabs was able to block HAVCR1 binding to HAV-coated ELISA wells.

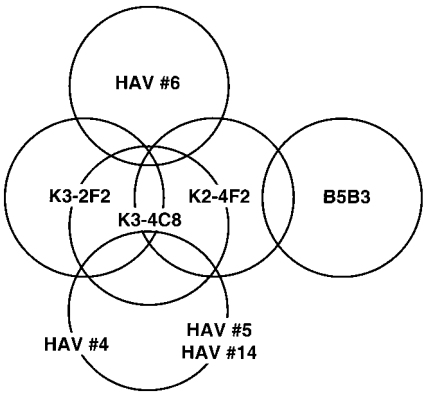

FIG. 7. The predicted topography of the epitopes recognized by four chimpanzee MAbs HAV#4, HAV#5, HAV#6, and HAV#14 within the context of the topography of epitopes characterized by mouse MAbs K3-4C8, K2-4F2, K3-2F2, and B5B3 (Ping *et al.,* 1988; Ping and Lemon, 1992; Stapleton and Lemon, 1987). Overlap between any two circles indicates >50% inhibition of binding. HAV#4, HAV#5, and HAV#14 epitopes are represented by a single circle; however, HAV#4 splits into a different group based upon its ability to neutralize the simian HAV strain, AGM-27.

was not neutralized by HAV#5 or HAV#14 means that one or more of the amino acids differentiating the capsid proteins of the HM-175 and AGM-27 strains must be critical for epitope recognition by these antibodies: unfortunately this narrows the possibilities only down to 27 amino acids after the VP3-070 amino acid is excluded.

HAV#6 neutralized both the HM-175 VP3-070 mutant virus and the simian HAV strain AGM-27. Therefore, HAV#6 appears to recognize a novel epitope on the HAV capsid that is not defined by any of the amino acid differences between HM-175 and AGM-27. In future work we hope to determine if any amino acid mutations allow escape from neutralization by the four chimpanzee MAbs. However, it is possible that no amino acid mutations will be identified if a mutation in a particular epitope renders the virus nonviable.

It is conceivable that the two sets of antibodies (murine and chimpanzee) are directed to distinct epitopes on the HAV capsid because of differences in antigen processing by the mouse and chimpanzee antigen-presenting cells as has been suggested for poliovirus (Uhlig and Dernick, 1988). However, competition studies with murine anti-HAV MAbs and anti-HAV positive human sera suggest that this is not the case for HAV (Ping and Lemon, 1992). Our results may also reflect differences in the affinities for the HAV capsid of these two sets of MAbs (murine and chimpanzee). If one antibody in the pair had a significantly higher affinity for the HAV capsid than did the other, then the lower affinity antibody may not have been able to compete successfully for binding to the capsid even if it recognized the same epitope. Due to the large amount of highly purified virus needed to determine antibody affinities, we have been unable to address this question.

Recently it has been observed that antibodies to some picornaviruses are able to neutralize virus infectivity by inhibiting the virus–cell receptor interaction (Smith *et al.,* 1996). However, little is known about the mechanisms by which antibodies neutralize hepatitis A virus. We examined whether our neutralizing MAbs were able to inhibit the binding of the soluble simian cell HAV receptor, recently isolated by Kaplan *et al.* (1996), to HM-175-coated wells. In indirect competition assays between the soluble receptor and each of the chimpanzee MAbs (as IgG or Fab), neither was able to inhibit the other from binding to HM-175-coated wells. Therefore, it seems unlikely that these antibodies neutralize virus infectivity by inhibiting virus attachment to cells via this receptor. The actual mechanism(s) by which these antibodies neutralize the virus remains undetermined. However, since the antibodies neutralized as Fab fragments, it is clear that bivalent binding is not necessary for neutralization.

Convalescent human anti-HAV, in the form of normal immune globulin, has been used for decades as a preexposure and postexposure immunoprophylactic agent against hepatitis A. In recent years its use has diminished because of its unavailability and because licensed hepatitis A vaccines have replaced it for most preexposure applications. However, vaccination requires a minimum of 2 weeks to achieve protection and postexposure prophylaxis therefore continues to be a legitimate application for immune globulin. In addition, there are other potential uses for such globulin: fulminant hepatitis A has been reported to account for up to 10–20% of liver transplants in children in some countries (Ciocca, 2000) and to be more common in adults who have coexisting liver disease (Lefilliatre and Villeneuve, 2000; O'Grady, 2000; Vento, 2000). Also, although normally self-limiting, HAV infection can cause persistent or relapsing hepatitis, especially in those who are immunosuppressed; recurrent hepatitis A has been reported following liver transplantation for fulminant disease (Gane *et al.,* 1995). This has prompted the suggestion that HAV-specific immune globulin be given at the time of transplantation for HAV-induced acute liver failure to prevent such recurrences (Gane *et al.,* 1995). Thus, there continue to be potential clinical applications for a broadly reactive and potent hepatitis A immune globulin. It will be interesting to determine the efficacy of immunoglobulin derived from the MAbs described herein for preventing and possibly treating hepatitis A.

In conclusion, we have characterized four chimpanzee MAbs to the HAV capsid, which were generated from a library of antibody genes amplified by PCR with human heavy- and light-chain gene-specific primers. Our competition data with previously well-characterized murine MAbs seem to be in accordance with the earlier studies that suggest there is a single immunodominant antigen site on the HAV capsid. Our MAbs competed with one of the murine MAbs that were used to define this site.

However, at least three of our chimpanzee MAbs appear to be directed to epitopes as yet undefined by previous studies. Thus one might speculate that although there are no gross differences in antigen presentation between the murine and primate systems there may be subtle differences in how antigens are presented to the two immune systems.

MATERIALS AND METHODS

Animals

Chimpanzee 1441 had been experimentally infected with the HAV strain, HM-175. Bone marrow was aspirated from the iliac crest of this animal 6 years postinfection. Three weeks prior to the bone marrow aspiration, the animal was boosted with a single dose of the inactivated HAV vaccine, HAVRIX (SmithKline Beecham), which contains the HM-175 capsid. The animal was maintained in an approved facility under conditions that met or exceeded all requirements for animal use.

Construction of 1/κ antibody phage library

The library construction was described in detail in Schofield *et al.* (2000).

Panning and ELISA reagents

Fabs were detected by enzyme-linked immunosorbant assays (ELISA) with goat anti-human IgG (H+L)-specific antibody (Pierce). This was coated on EIA/RIA A/2 plates (Costar) overnight at 4°C at a dilution of 1:1000, in 50 mM sodium carbonate buffer (pH 9.6). In all panning experiments and other ELISAs, formalin-inactivated HAV particles (HM-175 strain; Viral Antigens Inc.) were diluted 1:2 in 50 mM sodium carbonate buffer (pH 9.6) and coated on microtiter wells as above.

Library screening

Screening of the combinatorial library was carried out as described previously (Barbas *et al.,* 1991; Schofield *et al.,* 2000; Williamson *et al.,* 1993). Phages were selected by three rounds of panning on HM-175-coated ELISA wells. After amplification of the selected library, the phagemid DNA was extracted and modified by restriction enzyme digestion to allow soluble Fab expression in *Escherichia coli.* Single colonies were picked; Fab production was induced as described previously (Glamann *et al.,* 1998), and the bacterial supernatants were tested by ELISA for reactivity with HAV virions and for the presence of Fab.

Fab production, purification, and biotinylation

Bacterial culture and Fab fragment purification were carried out as described by Glamann *et al.* (1998). Protein concentrations were determined both by dye binding assay (Bio-Rad) and by A280 nm (using the extinction coefficient of 1.4 optical density units equivalent to 1.0 mg ml^{-1}). The Fab purity was determined by polyacrylamide gel electrophoresis followed by colloidal Coomassie blue staining (Sigma). The purified Fabs were diluted in sodium bicarbonate buffer (pH 9.0), and biotinylated at 4°C as per the manufacturer's protocol (Pierce). After biotinylation, the Fabs were dialyzed against phosphate-buffered saline (PBS) (pH 7.4) overnight at 4°C and concentrated in Centricon-30 concentrators (Amicon) as required.

ELISA analysis of Fab specificity

HAV was coated on ELISA microtiter plates at a 1:2 dilution and nonspecific proteins (thyroglobulin, lysozyme, glyceraldehyde-3-phosphate, chicken albumen, and cytochrome C (Sigma)) were coated at 10.0 μg ml^{-1}. ELISAs were performed as detailed previously (Schofield *et al.,* 2000).

Restriction digestion analysis and nucleic acid sequence analysis of HAV-specific Fab clones

For *Bst*N1 (New England Biologicals) fingerprinting, 1 μg of plasmid DNA was digested with 1 U of enzyme overnight at 60°C. The restriction digests were analyzed on a 3% agarose gel. Nucleic acid sequencing was performed with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with Ampli-Taq DNA Polymerase (Perkin–Elmer) and the sequencing primers HC1 and HC4 for the heavy-chain and LC1 and LC4 for the κ-chain (Glamann *et al.,* 1998). Sequences were analyzed with the GeneWorks (Oxford Molecular Group) software package. Sequence similarity searches were performed with the V-BASE program, which is a compilation of all the available human variable segment Ig germ line sequences (Cook and Tomlinson, 1995).

Radioimmunofocus assay

A modified radioimmunofocus assay was performed as described by Raychaudhuri *et al.* (1998). Briefly, chloroform-extracted HAV was mixed with antibody diluted in 10% BSA/PBS and incubated for 2 h at room temperature. Log_{10} dilutions of the virus–antibody mixtures were inoculated onto confluent monolayers of fetal rhesus kidney (subclone 11-1) cells grown on 25-mm-diameter Thermonox plastic coverslips (fixed to the bottom of each well) for 1 h at 34.5°C in a CO_2 incubator. The infected cells were then overlaid with 0.5% agarose–MEM supplemented with glutamine, gentamycin, and 2% FCS and incubated for 10–14 days at 34.5°C. The cell monolayers were fixed with acetone, air dried, and stored at −20°C until stained. HAV foci were reacted with a 1:500 dilution of chimpanzee 1442 antisera for 30 min at 34.5°C. The cells were washed three times in PBS–glycine and then incubated with ^{125}I-labeled sheep anti-human IgG $F(ab')_2$

fragment (1.0 μCi/ml; Amersham) for 1 h at 34.5°C. After four washes, the coverslips were air dried and exposed to X-ray film (Kodak) at −70°C.

Epitope mapping by indirect competition ELISA

The competing MAbs were titrated on HM-175-coated wells to determine the dilution that gave an OD reading of approximately 1.0 at $A_{405\,nm}$ and did not saturate the antigen coated to the plate. For the competition assay, threefold dilutions of unlabeled MAbs were incubated in HM-175-coated wells for 1 h at 37°C and then washed four times with PBS-Tween 20. A single dilution of the competing MAb (biotinylated Fab, whole IgG, or mouse IgG) was incubated in all wells for 1 h at 37°C. After four washes with PBS–Tween 20, the binding of the competitor MAb was detected with either streptavidin–alkaline phosphatase (Pierce), anti-human IgG (Fc-specific) alkaline phosphatase-conjugated antibody (Sigma), or anti-mouse IgG (H+L chain-specific) alkaline phosphatase-conjugated antibody (Pierce).

Conversion of Fab to whole IgG molecules and expression in COS-7 cells

The heavy- and light-chain genes of the MAbs were subcloned into pFab-CMV (a kind gift from Dr. Pietro Sanna (Sanna *et al.,* 1999)) using the same restriction enzyme sites used to clone the heavy- and light-chain genes into the phage display vector, pComb3H. After confirming the sequence of each clone, whole IgGs were obtained from tissue culture supernatants 5 days post-transfection of confluent COS-7 cells in T-75 flasks (Costar). Transfection of the plasmid DNAs was performed with Superfect transfection reagent (Qiagen) according to the manufacturer's instructions.

Cell receptor-blocking assay

A range of dilutions of the soluble simian cell receptor for HAV, HAVCR1 (kindly provided by Dr. Gerardo Kaplan (Silberstein *et al.,* 2001)), was incubated in HM-175-coated wells for 1 h at 37°C. After washing, a constant amount of MAb was added to the wells and incubated for 1 h at 37°C. The bound antibody was detected with an anti-human IgG Fab-specific alkaline phosphatase-labeled secondary antibody (1:5000). The optical density was measured at 405 nm with a reference wavelength of 650 nm. Reciprocal assays were also performed with dilutions of the anti-HAV antibodies and constant amounts of HAVCR1. HAVCR1 was generated as a fusion protein with the human IgG Fc region, and binding was detected with an anti-human IgG (Fc-specific) alkaline phosphatase-labeled secondary antibody (1:5000).

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