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Review Article

Four chimpanzee monoclonal antibodies that neutralize hepatitis A virus+

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Abstract

Chimpanzee immunoglobulins are virtually identical to human immunoglobulins and thus may have clinically useful applications. In a recent manuscript we described 4 chimpanzee monoclonal antibodies (MAbs) that neutralized hepatitis A virus (HAV) (1). The MAbs were isolated from a combinatorial cDNA library of chimpanzee gamma1/kappa antibody genes using bacteriophage displaying Fab fragments on their surfaces. Three of the MAbs recognized the same or overlapping epitopes on the HAV capsid, whereas the fourth recognized a different, nonoverlapping epitope on the capsid. All 4 MAbs neutralized the homologous HAV strain, HM-175, in a radioimmunofocus assay and 2 of the 4 MAbs neutralized a heterologous simian HAV strain, AGM-27. From these data, we conclude that the MAbs must recognize at least 3 epitopes on the HAV capsid. Competition assays performed with neutralizing murine MAbs suggested that 3 of the chimpanzee MAbs recognized epitopes on the HAV capsid which have not been defined previously. It is hoped that these MAbs may be used directly in passive immunoprophylaxis to prevent hepatitis A in susceptible populations, *e.g.*, travelers to regions where HAV is endemic or at risk individuals where epidemics are occurring.

Introduction

Hepatitis A virus

The 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. Indeed, prior to the availability of the current inactivated virus vaccines, pooled human immune globulin preparations were used routinely to protect individuals traveling to areas of the world where hepatitis A is endemic (2).

The virus has an icosahedral structure that is comprised of pentamers of 3 proteins, VP1, VP2 and VP3. Epitopes recognized by neutralizing antibodies on the HAV capsid are dependent on the conformation of the antigen (2). Mapping of antibody-neutralization escape mutants has identified an immunodominant site comprising amino acids from both the VP1 and VP3 proteins. Furthermore, only a relatively small number of amino acid mutations accounts for escape of these mutants from neutralization by most of the neutralizing murine MAbs available (3).

cDNA antibody libraries and antibody phage display

The display of functional antibody fragments on the surface of filamentous phage particles has provided a powerful tool for the generation of human MAbs to a variety of infectious agents such as human immunodeficiency virus type 1 (HIV-1)(4), hepatitis C virus (HCV)(5), Ebola virus (6), as well as to cancer markers, *e.g.*, melanoma (7), adenocarcinoma (8), ovarian carcinoma (9). Generally, for viral pathogens, naturally infected human donors have been used as the source of bone marrow cells or peripheral blood lymphocytes for the construction of these libraries. A number of very large “naive” libraries have been constructed from B cells collected from uninfected donors (10) or have been generated synthetically (11), however, very few antiviral antibodies have been produced from these “naive” libraries, to date.

+Sequence data from this article have been deposited with EMBL/GenBank Data Libraries under Accession Nos. AF411913-AF411916.

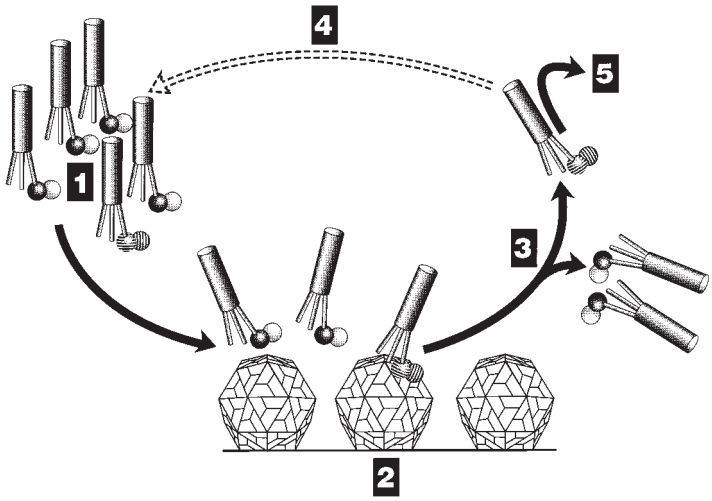

Fig. 1. An outline of the panning procedure. 1) The cDNA library of antibody genes in *E. coli* cells is displayed upon the surface of M13 bacteriophage following infection with helper phage. 2) The Fab-phage library is incubated on HAV coated to a solid support to select for antigen-specific binders. 3) Stringent washing removes nonspecific and low affinity Fab-phage binders, then the remaining bound Fab-phages are eluted from the antigen. 4) HAV-specific Fab-phage are amplified in fresh *E. coli* and the panning procedure is repeated twice more. 5) Following the final panning step the HAV-specific Fab-phage are eluted and amplified in *E. coli*. The DNA encoding the binding Fabs is extracted and modified by restriction enzyme digestion to allow production of soluble Fab for biological and biochemical characterization.

MAbs produced from human antibody gene libraries have the potential to serve directly as immunoprophylactic or therapeutic reagents against both infectious agents and cancer.

The advantages of using a chimpanzee as a donor for repertoire cloning are 2-fold. First, that chimpanzees can be readily infected experimentally by many of the important human viral pathogens, *e.g.*, HIV-1, HCV, hepatitis B virus (HBV) and respiratory syncytial virus (RSV). In some cases, chimpanzees are the only model for the specific human pathogen (*e.g.*, HCV). Second, the chimpanzee is the primate most closely related to humans. Therefore, chimpanzee antibodies could, theoretically, be used directly in the immunoprophylactic treatment of infectious diseases. A number of publications have indirectly addressed the possibility of using primate reagents in human prophylaxis and therapy by introducing human immune components into primates (12-18). These data show that little immunogenicity is seen when human immune components are introduced into chimpanzees compared to other nonhuman primates. Human antibodies apparently are recognized as self by the chimpanzee immune system. For example, the half-life of a human MAb was equivalent in a chimpanzee to the estimated half-life of IgG in humans (17). Hence, one would anticipate that chimpanzee antibodies would not be immunogenic in humans and so would have the same advantages as human antibodies for clinical applications, *i.e.*, without the need for modification (such as “humanization” required for antibodies from other species like the mouse).

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

The combinatorial antibody library described herein was generated from a chimpanzee that had been experimentally infected over a period of several years with the 5 recognized hepatitis-causing viruses, hepatitis A, B, C, D and E viruses. The chimpanzee was seropositive for antibodies to all 5 viruses. Previously, we used this antibody library to isolate neutralizing MAbs to the hepatitis E virus capsid protein (19). Subsequently, we have also isolated antibodies to hepatitis B and hepatitis D viruses from this library (Schofield *et al.*, unpublished data). In the Schofield *et al.* (1) study, we used inactivated whole HAV particles to isolate 4 MAbs 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.

Results and discussion

In this analysis of the library, 4 MAbs directed to the HAV capsid were identified. The selection of antigen-specific antibodies utilized a process termed “panning” which is outlined in Figure 1. After 3 rounds of panning on HAV particles, 300 clones were analyzed for HAV specificity by ELISA. The heavy chain nucleotide sequences of the HAV-specific clones were determined and four unique gamma1-heavy chains, HAV#4, HAV#5, HAV#6 and HAV#14, were identified (Table I). The gamma1-heavy chains of the HAV MAbs were most closely related, at the

Table I: Amino acid sequence data for the complementarity determining region 3 (CDR3) of the gamma1-heavy chain of the four HAV-specific MAbs and their classification based on nucleotide sequence homology with human immunoglobulin germ line genes.

MAb	V_H CDR3 sequence	V_H family	V_H segment	D segment	J_H segment
HAV#4	DLPGTWNFVDVFDI	CH1	DP-25[1]	D1-7[2]	JH3b
HAV#5	ASYGNYNYFYNMDV	VH5	DP-73[1]	D4-11[2]	JH6c
HAV#6	VFQSKGGAFWAPTTEWTYSYYYYMDV	VH4	DP-78[3]	D1-1[2]	JH6c
HAV#14	STIGVWDYYYYMDV	VH5	DP-73	ND	JH6c

ND: not determined due to lack of identifiable homologue. [1]Ref. 32, [2]Ref. 33, [3]V-BASE database.

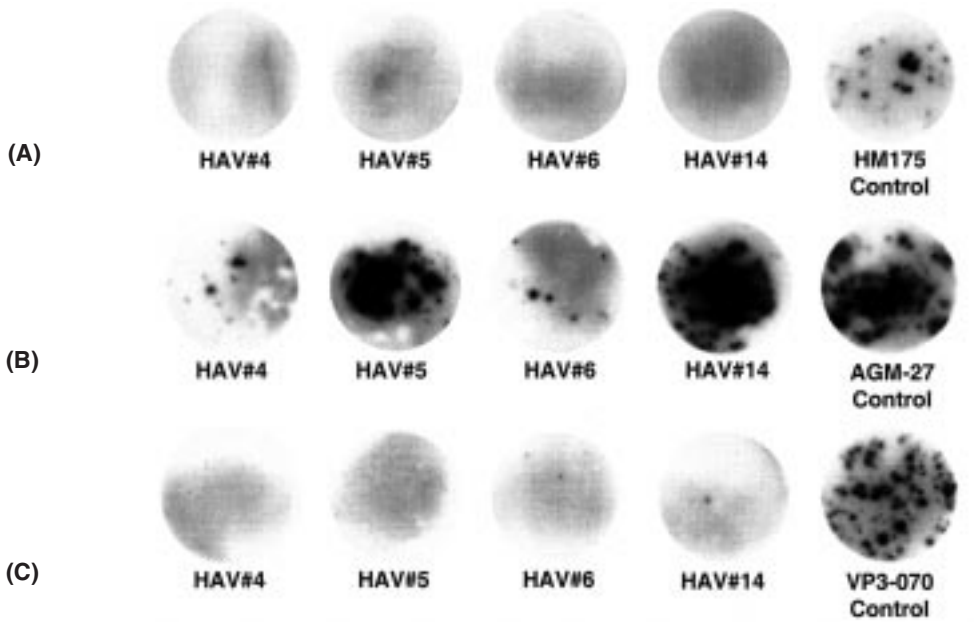

Fig. 2. RIFA assay of neutralization of HAV. An absence or decrease of foci relative to the control indicates neutralization. (A) Chimpanzee Fabs HAV#4, HAV#5, HAV#6 and HAV#14 neutralized the HM-175 strain of HAV. (B) Chimpanzee Fabs HAV#4 and HAV#6 neutralized AGM-27, however, chimpanzee Fabs HAV#5 and HAV#14 did not. (C) Chimpanzee Fabs HAV#4, HAV#5, HAV#6 and HAV#14 neutralized this HM-175 VP3 $Asp_{70} \rightarrow Ala_{70}$ mutant virus.

nucleotide level, to gamma1-heavy chains from the human VH1, VH4 and VH5 gene families (Table I). Indeed, the library was constructed using human V_H and V_κ gene family-specific primers, further indicating the close homology between the 2 species. Moreover, such chimpanzee-derived immunoglobulin sequences differ from human-derived sequences no more than genetically distinct human sequences differ from each other. Furthermore, as human-derived immunoglobulins exhibit the same half-life in chimpanzees as they do in humans, this would suggest that human antibodies are recognized by chimpanzees as self and not foreign antigens.

Competition assays suggested the MAbs recognized more than one epitope on the HAV capsid since 3 MAbs competed strongly with each other (HAV#4, HAV#5 and HAV#14) for binding but the fourth (HAV#6) competed poorly. All 4 MAbs neutralized the homologous HAV strain, HM-175, in a radioimmunofocus assay (RIFA) (Fig. 2a). This was the same strain used as the panning antigen, and was also the strain used to infect the chimpanzee from which the bone marrow was obtained. Two of the 4 MAbs also neutralized the divergent AGM-27 strain and 2 did not (Fig. 2b). AGM-27 is one of the most divergent HAV strains identified thus far (20).

These data, taken with the neutralization assay data, indicated that there were at least 3 different epitopes recognized by the 4 MAbs. Of the 3 MAbs that 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 2 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 also performed with the 4 chimpanzee MAbs, as both Fabs and whole IgG

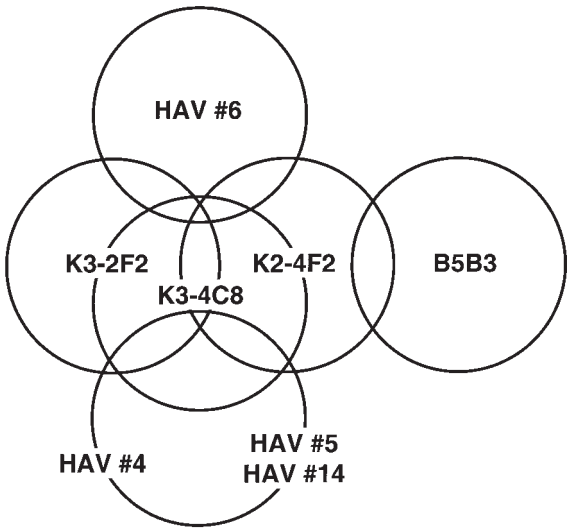

Fig. 3. The predicted topography of the epitopes recognized by four chimpanzee MAbs HAV#4, HAV#5, HAV#6 and HAV#14 within the context of the topography of epitopes characterized by mouse MAbs K3-4C8, K2-4F2, K3-2F2 and B5B3 (3, 22, 23). Overlap between any two circles indicates > 50% inhibition of binding. HAV#4, HAV#5 and HAV#14 epitopes are represented by a single circle, based on competition assay data, however HAV#4 is different from HAV #5 and HAV #14 based upon its ability to neutralize the simian HAV strain, AGM-27.

molecules, and with the mouse MAbs K3-2F2, K3-4C8, K2-4F2 (21), and B5B3 (Biogenesis).

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 (3, 22, 23) (Fig. 3). 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 II) suggested that MAb K3-4C8 and HAV#4 either recognized the same epitope or 2 epitopes that overlap extensively. In contrast, the data overall suggested that the other 3 chimpanzee MAbs (HAV#5, HAV#6, HAV#14) recognize epitopes different from those recognized by the majority of neutralizing murine MAbs. Mapping of neutralization escape mutants has implicated VP3-070, VP1-102, and VP1-221 as critical for neutralization by the murine MAbs. However, it can be presumed that these amino acids are not components of the epitopes recognized by the chimpanzee MAbs. Indeed, VP3-070 was experimentally excluded since all 4 chimpanzee MAbs neutralized a HM-175 VP3-070 mutant (Fig. 2c) and 2 MAbs, HAV#4 and HAV#6, neutralized the AGM-27 virus which also has the VP3-070 mutation. The fact that AGM-27 was not neutralized by HAV#5 or HAV#14 means that one or more of the remaining 27 amino acids differentiating the capsid proteins of the HM-175 and AGM-27 strains must be critical for epitope recognition by these antibodies.

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 4 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 2 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 (24). However, competition studies with murine anti-HAV MAbs and anti-HAV positive human sera suggest that this is not the case for HAV (3). Our results may also reflect differences in the affinities of these murine and chimpanzee MAbs for the HAV capsid. 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

Table II: 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.

Antibody	Neutralization			Epitope mapping[+]			
	HM-175	VP3-070	AGM-27	HAV#4*	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[+]	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

ND: not determined. *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. [+]"Yes" is defined as >60% inhibition of binding to HM-175-coated ELISA wells for each antibody pair.

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 (25). However, little is known about the mechanisms by which antibodies neutralize HAV. We examined whether our neutralizing MAbs were able to inhibit the binding to HAV of the soluble simian cell HAV receptor, recently isolated by Kaplan *et al.* (26). 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 HAV-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 pre- 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 (27) and to be more common in adults who have coexisting liver disease (28-30). 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 (31). 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 (31). 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.

With the continued and increasing prevalence of HIV-1 throughout the world, and the growing concerns over the transmissibility of prion diseases, such as Creutzfeld Jacob disease, from human blood-derived or tissue-derived materials, it seems inevitable that immune globulins derived from human blood will become unacceptable for use in the prevention of infectious diseases in the future. Therefore, it would seem highly likely that production of immunoglobulins by recombinant techniques will become routine. At present, both cost and lack of fermenter capacity within the industry make this approach prohibitively expensive. However, as technology advances, recombinant protein use in the clinic will become more prevalent. Therefore, antibodies such as those described here may well be a valuable resource in these future applications. Although these Fab fragments were derived from a chimpanzee, albeit amplified using human antibody gene family-specific PCR primers, their conversion to whole IgG molecules for expression in mammalian cells will also humanize these antibodies further. The Fc components supplied by such whole IgG expression vectors are derived from human IgG antibody genes. It is very unlikely that a nonself immune response to such chimpanzee-human chimeric antibodies will occur. However, as with all human MAbs, each chimpanzee whole IgG will have to be examined individually for immunogenicity and safety in human volunteers.

Conclusions

We have characterized 4 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 3 of our chimpanzee MAbs appear to be directed to epitopes as yet undefined by previous studies.

In future studies, we hope to show that these MAbs, which neutralize HAV *in vitro*, are capable of protecting chimpanzees from an HAV challenge *in vivo*. If this occurs it would strongly suggest that these MAbs may have a role as a passive immunoprophylactic reagent.

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