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Human monoclonal antibody that recognizes the V3 region of human immunodeficiency virus gp120 and neutralizes the human T-lymphotropic virus type III_{MN} strain

(AIDS/human immunodeficiency virus type 1/virus neutralization/epitope mapping)

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ABSTRACT **We describe a human IgG1 monoclonal antibody (N701.9b) derived by Epstein–Barr virus transformation of B cells from a human immunodeficiency virus-seropositive asymptomatic donor. This antibody was shown to recognize the principal neutralizing domain contained within the V3 region of gp120 of the MN strain of human immunodeficiency virus and MN-like strains, as determined by binding to the PB-1 fragment of MN gp120 and to synthetic peptides corresponding to the V3 region of MN and related virus strains. The epitope identified by monoclonal antibody N701.9b was mapped to a segment of V3 containing at least 7 amino acids (amino acids 316–322), which is located in the "tip" and "right" side of the V3 loop of the MN strain. Furthermore, this antibody manifested potent type-specific fusion-inhibitory activity against the MN strain but not against the IIIB or RF virus strains. This antibody also neutralized four virus isolates that had MN-like V3 region sequences and failed to neutralize three other strains containing unrelated V3 region sequences. Our findings confirm that the V3 region stimulates type-specific neutralizing antibody during natural human immunodeficiency virus infection in humans. The potential clinical use of this antibody is discussed.**

Human immunodeficiency virus type 1 (HIV-1) productively infects human $CD4^+$ cells, including $CD4^+$ T cells, cells of the monocyte/macrophage lineage, and other cell types, such as neuronal cells (1, 2). Infection with HIV-1 leads, in most cases, to a progressive decline in the number and functions of $CD4^+$ T cells with the eventual appearance of clinical manifestations of cellular immunodeficiency, such as opportunistic infections and malignancies—i.e., AIDS (3). Immune responses to HIV-1 in infected hosts have been well documented and include development of antibodies to a variety of viral proteins (4–7) and cellular, especially cytotoxic, responses to viral protein-expressing cells (8–10). In particular, antibodies that neutralize HIV develop concomitantly with seroconversion, and in most HIV-infected individuals such antibodies can neutralize a wide variety of strains of HIV, a virus noted for extraordinary variability in the amino acid sequence of its external glycoprotein, gp120 (11–13).

The precise proteins and epitopes on HIV proteins that elicit neutralizing antibodies have been well studied in experimental animals immunized with purified viral proteins, recombinant protein fragments, and synthetic peptides (14–22). Although a variety of epitopes on gp120 and gp41 have been associated with the ability to induce neutralizing antibodies in certain animal strains, most workers in the field agree that the highly variable V3 region of gp120, the so-called "loop," is a particularly immunodominant site for inducing neutralizing antibodies (17, 18, 21). Recombinant protein fragments containing the V3 loop and synthetic peptides corresponding to a 24-amino acid stretch encompassing amino acids 307–330 contained within the V3 region have been shown to induce type-specific neutralizing antibodies in several animal species (17, 18, 21, 22). More recently a 9-amino acid portion of the V3 loop, which contains the relatively conserved Gly-Pro-Gly-Arg (GPGR) sequence and may constitute a β-turn structure, has been determined as the actual site recognized by these type-specific neutralizing antibodies (19, 23). The 24-amino acid sequence within the V3 region is believed to represent the principal neutralizing domain (PND) of HIV. This idea is supported by the observation that most rodent monoclonal antibodies (mAbs) produced so far, which have consistent HIV-neutralizing properties *in vitro* and for which the binding site is known, recognize this epitope (24–28).

Whether the V3 loop represents the PND in natural HIV infection in humans is not as certain. Part of the difficulty in making this determination arises from the fact that the loop region of gp120 varies greatly among different strains of HIV, and until recently, whether any HIV strains actually represented dominant serotypes was unclear. However, the most common V3 genotype in North America appears to be more closely related to the human T-lymphotropic virus (HTLV) type III_{MN} family of HIV than the more commonly studied HTLV-III_B strain (from polymerase chain reaction-derived sequence analysis of the V3 region of several isolates) (23). These observations led our group to do studies in which human HIV^+ sera with high titers of neutralizing activity against HIV_{MN} were absorbed with peptides corresponding to the V3 loop of strain MN. Preliminary data suggested that most MN-directed neutralizing antibodies recognize the V3 loop, although significant amounts of neutralizing activity may be directed to other epitopes (29). In addition, observations of the neutralizing activity of sera from a laboratory worker accidentally infected with the III_B strain suggest that during initial stages of infection most neutralizing antibodies are V3-loop directed (30).

In this report, we have attempted to define more precisely the human serologic response to HIV by characterizing human mAbs derived by Epstein–Barr virus transformation of human B cells from an HIV-seropositive donor (31). We

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Abbreviations: HIV-1, human immunodeficiency virus type 1; HTLV-III_B, -III_{MN}, -III_{RF}, human T-cell lymphotropic virus type III, isolates B, MN, and RF, respectively—now designated as the IIIB, MN, and RF strains of HIV-1; PND, principal neutralization domain of HIV-1; mAb, monoclonal antibody; vac-gp160, recombinant vaccinia virus expressing HIV gp160; ADCC, antibody-dependent cell-mediated cytotoxicity.

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demonstrate that a gp120-specific human mAb that has significant HIV-neutralizing activity can be derived from such B cells and that the epitope recognized by this antibody is within the V3 region of HIV_{MN}.

MATERIALS AND METHODS

Derivation of Human mAbs. The derivation and preliminary characterization of three human mAbs, designated N701.9b, N701.5e, and N702.3a, respectively, derived from a single B-cell donor, have been described in detail (31). mAbs N701.5e and N702.3a bind to relatively conserved, conformational determinants on gp120, whereas mAb N701.9b is much more strain-restricted in its recognition pattern, reacting only with gp120 from MN, SF-2, and J62 strains (31).

Peptide Synthesis. Peptides were synthesized using an Applied Biosystems synthesizer, model 431.

Cell Lines. $CD4^+$ CEM cells are derived from a line obtained from the American Type Culture Collection (ATCC CCL 119). They were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, L-glutamine, and antibiotics.

Recombinant Vaccinia Virus-Expressing HIV gp160. Vaccinia virus recombinants expressing the complete envelope (*env*) genes of HIV_{IIIB} and HIV_{RF} were provided by Bernard Moss (Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases), the construction of which has been described (32). Recombinant virus expressing the PND of HIV_{MN} was constructed by replacing the 580-base-pair *Bgl* III DNA fragment at pSC25 (the coexpression vector containing the gene for gp160 IIIB) with the *Bgl* III DNA fragment isolated from the *env* gene of the MN variant. The resulting coexpression vector, designated pSCR2502, contains the DNA sequence coding for amino acids 278–468 of the MN envelope, within which lies the PND (17). Transfection isolation of virus recombinants was done as described (33).

Syncytia Inhibition Assay. Recombinant vaccinia virus expressing HIV gp160 (vac-gp160) at a multiplicity of infection of 1 was allowed to infect CEM cells for 1–2 hr, after which excess virus was removed. Syncytia were apparent at 24 hr after infection but were never seen after infection with vaccinia virus expressing the product of the α-galactosidase gene but not gp160 [vsc8 (32)]. These vac-gp160-induced syncytia were inhibited by antibodies directed to the PND of gp160 (see Table 2), as well as by OKT4A antibody and recombinant gp120 (S.S., unpublished observations). The titer of antiserum or the concentration of mAb necessary to inhibit 90% of syncytia in this assay correlated well with that necessary to reduce reverse transcriptase activity by 50% in an HIV-based assay of neutralization (S.S., unpublished data). To test the efficacy of mAbs in blocking syncytia formation or of peptides in reversing that blockade, antibody at the concentrations or dilutions indicated in the text was added 1 hr after infection to culture wells, or peptide and antibody were admixed for 1 hr before addition to infected cells.

HIV-1 Field Isolates. Infectious HIV-1 was isolated from peripheral blood mononuclear cells of individual patients by standard cocultivation techniques using 72-hr normal phytohemagglutinin-derived T-cell blasts. Cultures received fresh phytohemagglutinin blasts at weekly intervals. HIV-1 was determined weekly by detection of p24 in cell-free culture supernatants by antigen-capture ELISA (DuPont). After attainment of peak p24 levels (i.e., >200 pg/ml), cell-free virus was transmitted to CEM cells, and infectious HIV pools were subsequently derived, measured, and stored at −70°C. Stocks were retitered as each neutralization assay was done. The V3 sequences of the seven HIV isolates used were determined and have been reported (23).

Neutralization Assay. Neutralization titers were determined as described (17). Briefly, 100 μl of 4-fold mAb dilutions were added to 100 μl of HIV stocks in 6-well plates and incubated for 30 min at 37°C. Virus antibody mixtures were incubated with 10^5 AA-5 (a $CD4^+$ Epstein–Barr virus-transformed B-cell line) target cells in 100 μl for 24 hr at 37°C, after which 3 ml of medium was added to each well. Cultures were terminated at 7 days after infection, and the supernatants were analyzed for reverse transcriptase activity. Neutralization titer was defined as the lowest concentration of antibody required to inhibit reverse transcriptase activity by 50%.

Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC) Assay. Peripheral blood mononuclear cells from an HIV-seronegative normal donor were incubated with the indicated concentrations of N701.9b and 5×10^3 ^{51}Cr-labeled CEM-NKR or CEM-NKR/MN targets, the derivation of which has been described (10). A standard effector-to-target cell ratio and a 6-hr assay incubation were used.

ELISA. Costar EIA plates coated with recombinant proteins at 50 μg/ml or with peptides at 2–5 μg/ml were blocked with a 0.5% bovine serum albumin solution and treated with antisera or mAbs for 2 hr. After being washed, plates were incubated with either goat anti-human Ig–HPRO or protein A–HPRO (Boehringer Mannheim), and color was developed using azinobis (3-ethyl benzthiazoline sulfonic acid) containing 0.03% H_2O_2. Optical density was read at 410 nm using a Multiscan plate reader (Dynatech).

RESULTS

The three human mAbs were first tested by ELISA for binding to V3 loop-containing PB-1 fragments of HIV gp120 (14, 17). mAb N701.9b reacted specifically with $PB\text{-}1_{MN}$, but not $PB\text{-}1_{IIIB}$ or $PB\text{-}1_{RF}$, whereas mAbs N701.5e and N702.3a did not bind to any of the PB-1 fragments (data not shown). These results suggested that mAb N701.9b reacted with a variable site within $PB\text{-}1_{MN}$, whereas mAbs N701.5e and N702.3a apparently recognized sites in a different portion of gp120. mAb N701.9b was also found to react by immunoblot assay with $PB\text{-}1_{MN}$ but not with $PB\text{-}1_{IIIB}$ or $gp160_{IIIB}$ (data not shown). Next, mAb N701.9b was tested for binding activity to synthetic peptides corresponding to V3 sequences of several HIV strains (Table 1). RP70 is a 40-amino acid peptide corresponding to the entire V3-loop region of the MN isolate and containing an intramolecular disulfide bond between the two conserved cysteine residues; RP142 and RP135 are homologous 24-amino acid linear peptides that correspond to the central portions of the V3 loop of MN and IIIB, respectively (17, 29). mAb N701.9b bound to both peptides RP70 and RP142, but not to peptide RP135, showing that this antibody binds to V3 of MN but not of IIIB. mAb N701.5e served as a negative control, as it did not bind to any of these peptides. To more precisely map the epitope identified by

Table 1. Binding of human mAb to V3 region peptides

	Antigen, OD_{410}			
Test reagent	RP70	RP142	RP135	Bovine serum albumin
HIV^- serum	0.120	0.139	0.159	0.083
HIV^+ serum	**0.308**	**0.735**	0.239	0.133
mAb N701.9b	**0.475**	**0.574**	0.223	0.164
mAb N701.5e	0.110	0.235	0.147	0.058

Sera were tested at 1:500; purified human mAbs were tested at 20 μg/ml. The sequences of V3 peptides (in one-letter code) are as follows: RP70, INCTRDNYNKRKRIHIGPGRAFYTTKNIIGTIRQAHCNIS; RP142, YNKRKRIHIGPGRAFYTTKNNIGC; RP135, NNTRKSIRIQRGPGRAFVTIGKIG. Boldface numbers indicate significant reactivity above background.

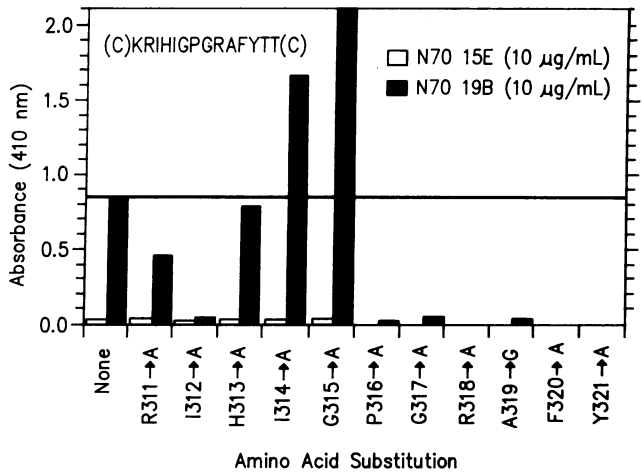

FIG. 1. Binding of mAbs N701.9b and N701.5e to peptide 310–323 and analogs containing single-amino acid substitutions. Direct ELISA was done on the parent peptide (C)KRIHIGPGRAFYTT(C) and peptides containing the indicated substitutions (one-letter code). Absorbances are the average of three determinations.

mAb N701.9b, we tested its ability to bind to a series of 14-amino acid peptides derived from the V3-MN-loop sequence, in which successive amino acids starting with amino acid 311 were replaced with alanine (Fig. 1). It was evident that the ability of mAb N701.9b to bind to the unsubstituted peptide was abrogated by alanine substitutions starting at Pro-316 and continuing to Tyr-322. An alanine substitution at Ile-312 also abrogated binding. These data indicated that mAb N701.9b bound to an epitope primarily defined by amino acids 316–322, or the so-called tip and right side of the V3 loop (19), although Ile-312 may also be involved in the binding site.

We then examined whether this V3-loop-directed antibody was functionally active in inhibiting HIV-induced fusion or in neutralization of cell-free HIV. First, we used an assay in which vac-gp160 induced syncytia formation in CEM cells, as described. These vac-gp160-induced syncytia are inhibited by V3-loop-directed antisera or V3-loop-directed murine mAbs in a type-specific fashion (Table 2). mAb N701.9b inhibited syncytia formation induced by vac-gp160$_{MN}$ but did not inhibit syncytia induced by vac-gp160$_{IIIB}$ or vac-gp160$_{RF}$ (Table 2). The endpoint concentration in the vac-gp160$_{MN}$-based assay was ≈0.5 μg/ml in three separate experiments (Table 3). Furthermore, a peptide-competition study, similar to those described (17), was done (Table 3). Peptide RP142, the MN-V3-loop 24-mer, but not peptide RP135, the IIIB-V3-loop 24-mer, abrogated the ability of mAb N701.9b to inhibit vac-gp160$_{MN}$-induced syncytia, confirming that the functional epitope recognized by mAb N701.9b indeed lay in the V3-MN loop. In addition, a 24-mer peptide corresponding to the PND of the SF-2 strain of HIV (peptide RP150), with which mAb N701.9b reacts (31), also abrogated the inhibition of syncytia. mAb N701.5e had no syncytia-inhibiting properties in the vac-gp160-based assay, although it has been shown to have neutralizing activity against a wide variety of strains in a standard HIV-infectivity assay (D. D. Ho, J. A. McKeating, X. L. Li, T. Moudgil, E. S. Daar, N.-C. Sun, and J.E.R., unpublished results). In another peptide competition experiment, V3-MN peptides with alanine substitutions in amino acids 317–322 (as used in Fig. 1) were unable to block the syncytia-inhibiting activity of mAb N701.9b, showing that the site involved in syncytia inhibition by mAb N701.9b was similar to the binding site identified by ELISA (data not shown).

Table 2. mAb inhibition of syncytia induced by gp160-expressing virus

Antibody	Induced syncytia, no. vac-gp160IIIB	vac-gp160MN	vac-gp160RF
None	79	>250	86
N701.9b	85	0	57
N701.5e	91	>250	47
Murine mAb 0.5β	0	ND	ND
Guinea pig anti-RP135 serum	0	>250	49
Guinea pig anti-RP142 serum	42	0	47
Goat anti-RP139 serum	75	>250	6

CD4$^+$ CEM cells were infected with vac-gp160 (vac-gp160IIIB, vac-gp160RF, or vac-gp160MN). mAbs were used at a final concentration of 10 μg/ml, and antisera were used at 1:40 dilution. Syncytia were scored 24 hr after infection. ND, not done.

Table 3. Endpoint titration and blocking of neutralization by soluble peptide

mAb N701.9b, μg/ml	Peptide (50 μg/ml)	Syncytia, no. Exp. 1	Exp. 2	Exp. 3
None		>200	78	>300
5		0	3	ND
2.5		0	4	ND
1		0	5	17
0.5		0	4	47
0.25		3	18	147
0.1		ND	33	>300
5	RP142	>200	71	
5	RP135	0	2	
5	RP150	ND	47	
5	RP151	ND	3	

Protocol was exactly as described in the legend for Table 2, except that the peptide was coincubated with antibody 1 hr before addition to infected cells. The sequence (in one-letter code) of RP150 is NNTRKSIYIGPGRAFHTTGRIIGC and that of RP151 is NNTKKGIAIGPGRTLYPREKIIGC.

We also examined the ability of mAb N701.9b to neutralize seven independent isolates of HIV, the V3 sequences of which are known (23), in a standard infectivity assay (17). Table 4 shows that mAb N701.9b neutralized four of the strains, including strain MN, at endpoint concentrations from 0.095 to 1.0 μg/ml. The other three strains were not neutralized at antibody concentrations as high as 20 μg/ml. There was a correlation between those viruses that contained the sequence Pro-Gly-Arg-Ala-Phe-Tyr-Thr-Thr (PGRAFYTT), representing the tip and right side of the loop, and those that were neutralized in this assay, providing further evidence that the binding site of mAb N701.9b was indeed on the right side of the V3 loop. Finally, we examined the ability of mAb N701.9b to mediate ADCC *in vitro* with HIV gp160-expressing targets. The results shown in Table 5 demonstrate that mAb N701.9b mediated the specific lysis of HIV$_{MN}$-infected CEM-NKR targets. Further experiments are required to determine whether the ADCC activity of mAb

Table 4. Neutralization of HIV by mAb N701.9b

Virus	V3-loop sequence	Neutralization endpoint, μg/ml
MN	RIHIGPGRAFYTT	0.90
DU 4489-5	RIPIGPGRAFYTT	0.095
DU 6587-3	RLSIGPGRSFYAT	>20
DU 6587-5	RIHIGPGRAFHTT	>20
DU 7887-7	GIRIGPGRAILAT	>20
DU 39188.1	RIPIGPGRAFYTT	0.44
DU 4489.4	RITIGPGRVFYTT	1.0

Table 5. Lysis of gp160-expressing target cells in ADCC assay

mAb N701.9b, μg/ml	Specific lysis, %	
	CEM-NKR	CEM-NKR/MN
100	1.2	18.8
4	2.3	11.7

Normal human peripheral blood lymphocytes were incubated at an effector-to-target cell ratio of 30:1 for 6 hr with the indicated concentrations of mAb N701.9b and 5×10^3 ^{51}Cr-labeled CEM-NKR or CEM-NKR/MN targets, the derivation of which has been described (14). Neither CEM-NKR nor CEM-NKR/MN was lysed in the presence of an irrelevant IgG1 (data not shown).

N701.9b parallels the strain specificity of its neutralizing activity.

DISCUSSION

Our observations have importance for several reasons. Several other groups have produced a number of human mAbs to HIV (34, 35), most of which recognized epitopes on either gp41 or p24 and not on gp120. None, so far to our knowledge, has had significant HIV-neutralizing activity *in vitro*. The human mAb N701.9b does identify an epitope within V3 of HIV gp120 and manifests unequivocal type-specific neutralizing and syncytia-inhibiting activity against HIV_{MN} and MN-like strains. The ability to derive mAb N701.9b from a naturally HIV-infected human also directly confirms that part of the neutralizing antibody repertoire in humans is V3-loop-directed. This mAb also allowed a more precise characterization of the epitope recognized by a human neutralizing antibody than has been possible by using human HIV^+ serum. Our findings indicate that mAb N701.9b binds to an epitope on V3-MN similar to that recognized on V3-IIIB by a described (24) murine mAb, 0.5β, and by a variety of rat anti-V3-IIIB neutralizing mAbs (C.F.S., unpublished work). The fact that mAb N701.9b is active against MN and MN-like strains of HIV and not against the more commonly studied IIIB strain correlates with the observation that natural infections occur more frequently with MN-like strains than with IIIB (24).

The availability of mAb N701.9b will allow precise analysis of the percentage of HIV isolates in the naturally infected human population serotypically related to the V3-MN family on their ability to be neutralized as opposed to determination of V3 relatedness based on "genotyping" by polymerase chain reaction sequence analysis. This is an important distinction because the amino acid sequence of V3 of any given virus isolate may not always correlate with its susceptibility to neutralization by an antibody that binds to the appropriate linear V3 sequence. Indeed, findings in a recent analysis of neutralization-resistant escape mutants selected *in vitro* by loop-directed murine mAbs suggest that significant changes in the conformation and function of V3 can result from single amino acid substitutions within or outside the V3 loop (36). Such conformational changes in V3 may diminish both the binding affinity and neutralizing activity of the selecting antibody against the variant virus. Although mAb N701.9b could neutralize four HIV isolates (Table 4), the endpoint concentration required to effect neutralization differed among these strains by as much as 10-fold, despite close similarities in their V3 sequences. The variable sensitivity of these strains to mAb N701.9b neutralization possibly is related to minor sequence differences within V3 or to some degree of conformational heterogeneity of V3 in these strains.

A number of questions remain concerning the nature of the neutralizing-antibody repertoire in HIV-infected humans. (*i*) To what extent do non-V3-loop epitopes on gp120 or gp41 induce neutralizing antibodies during natural infection? Preliminary studies using human HIV^+ antisera adsorbed with V3-loop peptides (29) or with recombinant envelope proteins (37) suggest inducement, although the precise epitopes involved are still unknown. Importantly, non-V3-loop neutralizing activity may be directed against discontinuous, conformational epitopes that are group-common (37). In this regard, the human mAb N701.5e, which was derived from the same B-cell donor as mAb N701.9b, has broad neutralizing activity, identifies a conformational epitope involved in gp120–CD4 binding, and is distinct from previously recognized functional epitopes of gp120 (D. D. Ho, J. A. McKeating, X. L. Li, T. Moudgil, E. S. Daar, N.-C. Sun, and J.E.R., unpublished results). Possibly, antibodies similar to N701.5e may explain the broadly neutralizing antibody activity found in most patient sera.

(*ii*) Are V3-loop-directed neutralizing antibodies always relatively type-specific in humans? It is possible that humans produce antibodies to regions of V3 that contain more conserved sequences, such as the central Gly-Pro-Gly-Arg-Ala-Phe (GPGRAF) motif, and such V3-directed antibodies may neutralize a wider panel of HIV strains. It would also be of interest to determine whether V3-directed neutralizing mAbs are primarily directed to the strains of HIV infecting each B-cell donor. Because it is now feasible to produce anti-gp120 human mAbs that have functional activity *in vitro* and that recognize either V3 or non-V3 neutralization epitopes, the production of large panels of human mAbs should provide essential reagents to further address these issues.

(*iii*) What is the potential clinical use of neutralizing human mAbs? Previous studies on the ability of neutralizing antibody to protect against viral challenge in chimpanzees have suggested that neither passively administered antibody nor antibody induced by vaccination offers protection (38–40). More recent experiments, however, suggest that V3-directed antibodies may be protective (41, 42); a study in which neutralizing mAb was admixed *in vitro* with virus before *in vivo* inoculation demonstrated at least partial protection (43). In addition, two groups have claimed that some clinical benefit was observed in patients with advanced HIV infection (i.e., AIDS) who were given plasma from healthy asymptomatic HIV^+ donors, which in one study contained high titers of neutralizing activity (44, 45). Thus, the potential exists for using human neutralizing mAbs, such as N701.9b, both for prophylaxis after exposure to HIV and for the treatment of advanced disease, although a mixture of human mAbs would be needed to provide neutralizing activity against a broad spectrum of virus strains. Human mAbs have an additional advantage over rodent or partially rodent (i.e., chimeric) mAbs in that they will be less immunogenic in humans (46). However, a more detailed analysis of the role that neutralizing antibody (or ADCC-mediating antibody) plays in slowing or halting the progression of HIV infection in humans is required, and the development of a panel of neutralizing human mAbs from a variety of HIV-infected donors may help resolve some of these questions.

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