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Neutralization of divergent human immunodeficiency virus type 1 variants and primary isolates by IAM-41-2F5, an anti-gp41 human monoclonal antibody

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ABSTRACT **The antiviral characteristics of monoclonal antibody IAM-41-2F5 (2F5) were determined in cell culture. The antibody had been previously shown to bind a specific sequence, ELDKWA, within the external domain of the gp41 envelope glycoprotein human immunodeficiency virus type 1 (HIV-1). Selection by 2F5 of recombinant phage from an epitope library confirmed the identification of the antibody's binding determinant. The antibody was found to be capable of neutralizing a broad range of lymphoid cell culture-adapted HIV-1 variants as well as HIV-1 primary isolates. Sequence analysis of the latter showed that neutralization was related to the presence of the antibody binding site. From kinetic measurements using an epitope-containing peptide or gp41, the half-time of dissociation for 2F5 was determined to be 122 min for the peptide and 156 min for gp41. The region of gp41 expressing this sequence exhibits greater conservation among HIV-1 isolates than do the variable domains of gp120.**

Insights into the development of a vaccine to prevent human immunodeficiency virus type 1 (HIV-1) infection may be obtained by the characterization of virus-neutralizing monoclonal antibodies (mAbs) and by the identification of viral determinants that bind the antibodies. To this end, a number of neutralizing human mAbs have been derived from HIV-1-infected persons. These antibodies generally belong to one of two neutralizing classes.

The first is directed to determinants expressed by the third hypervariable (V3) domain of the gp120 envelope glycoprotein. Antibodies in this class typically mediate potent neutralization of HIV-1 infection in cell culture, apparently by interference with an essential postreceptor binding event (1). Given the variable nature of the V3 domain (2), these antibodies are usually highly type-specific in their activity (3, 4). Nonetheless, anti-gp120 V3 antibodies, whether actively elicited or passively administered, are capable of protecting chimpanzees from HIV-1 challenge infection (5–7). However, immunogens designed to elicit anti-V3 antibodies have not successfully induced neutralizing activities that are sufficiently broad for practical application.

The second neutralizing antibody class is functionally directed to the viral gp120–CD4 binding site. These antibodies neutralize the virus by preventing gp120–CD4 interaction (8–11). Individual mAbs within this class appear to recognize distinct determinants on gp120 and can, therefore, be grouped into binding families (12). Mutational and structural studies of gp120 have shown that multiple discontinuous domains within the glycoprotein contribute to the CD4 binding site (13, 14). No immunogens have been reported that can consistently elicit virus-neutralizing antibodies that belong to this class; indeed, vaccines currently on test in humans have been shown not to elicit anti-CD4 binding site antibodies (15). Accordingly, the identification of additional viral determinants that can contribute to an HIV-1-neutralizing antibody response would be potentially useful for the design of vaccine immunogens.

Muster *et al.* (16) described the derivation of a virus-neutralizing human mAb, IAM-41-2F5 (2F5), directed to a previously unidentified neutralization determinant on the viral gp41 envelope-anchoring glycoprotein. To assess this determinant's potential, we have confirmed the binding site's identification¶ and characterized the antibody's virus-neutralizing activity. We report that antibody 2F5 has potent neutralizing activity directed against a broad range of divergent HIV-1 variants and primary isolates.

MATERIALS AND METHODS

Antibodies. Human mAb 2F5 was purchased from Virus Testing Systems (Houston). The antibody was prepared by Waldheim Pharmaceutika (Vienna). Human polyclonal serum 498 was obtained from an HIV-infected individual. This serum potently neutralizes most laboratory-adapted strains of HIV-1 (unpublished observations).

Preparation of Human Peripheral Blood Mononuclear Cells (PBMCs). Human PBMC cultures were prepared from fresh HIV-negative donor buffy-coat cells. The PBMC fraction purified in Ficoll/Hypaque gradients was collected and washed. The cells were cultured in RPMI 1640/20% fetal bovine serum and were stimulated with phytohemagglutinin (PHA-P, Sigma) at 5 μg/ml. After 48 hr, the cultures were expanded with additional medium and were supplemented with interleukin 2 (Boehringer Mannheim) at 40 units/ml. After an additional 3 days, the cultures were centrifuged and the cells were resuspended in fresh RPMI 1640/20% fetal bovine serum with interleukin 2 at 40 units/ml. Cells prepared in this manner were used for preparing stocks of primary virus isolates and for tests of antibody-mediated virus infectivity reduction.

Viruses. T-lymphoid-cell-adapted HIV-1 variant virus stocks were prepared from chronically infected H9 human T-lymphoid cell cultures (17). Virus stocks of the primary HIV-1 isolates designated MR452, UAB430, UAB431, UAB422, UAB112, and S060 were prepared by infection of 5×10^6 PBMCs. Extracellular virus and excipients from the inoculum were removed by centrifugation and resuspension in fresh medium on day 1 postinfection. An additional 15×10^6 PBMCs were added on day 2. On day 4, the cells were

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Abbreviations: HIV, human immunodeficiency virus; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cell.

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¶The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U06721–U06743).

again centrifuged and resuspended in fresh medium before the addition of 10×10^6 uninfected PBMCs. Virus was harvested and stock preparation was completed on day 7. The primary virus stock aliquots were stored in vapor-phase liquid N_2. Most of the virus in the stock was generated rapidly and accumulated in the culture medium between days 5 and 7 postinfection (unpublished observation). This method typically yielded a high-titer virus preparation.

***In Vitro* Virus Neutralization Assays.** Neutralization assays using established laboratory strains were performed with the MT-4 T-lymphoid cell killing assay (18). All neutralization end points were confirmed both by cell viability with the absence of cytopathic effect and by p24 antigen-capture ELISA (Coulter). Neutralization end points were defined as the concentration of mAb or dilution of serum that prevented cell killing and reduced p24 production by >90% compared with untreated controls. For *in vitro* neutralization tests using primary HIV-1 isolates in human PBMC cultures, an infectivity reduction format was used. By this method, the neutralization property of a constant mAb concentration or serum dilution was tested against a serial dilution series of primary virus stock. Virus and antibody were incubated for 1 hr at 37°C before addition of 5×10^4 PBMCs to each mixture. After 7 days in culture, virus replication was measured by p24 antigen ELISA and the reduction of infectivity determined by comparison of the replicative ability of each input concentration of virus in the presence and absence of the test antibody. Since each infectivity reduction test required a titration of the primary virus stock, there was an inherent check on both the replication of the virus stock and the capacity of the PBMC culture used to support that replication.

Extraction of RNA from Plasma and Virus Stocks and DNA Sequencing. To determine variability in the region of gp41 recognized by antibody 2F5, sequences of the *env* gene encompassing envelope glycoprotein residues 627–673 were determined for the primary virus isolates used in this study. Viral RNAs from each patient's plasma at the time of initial virus isolation also were analyzed. Plasma or virus stock was mixed with an equal volume of 5 M guanidine thiocyanate/0.5% *N*-lauroylsarcosine/0.1 M 2-mercaptoethanol/25 mM sodium citrate, pH 7.0. Sodium acetate (0.2 M) and tRNA (100 μg) were added and the solution was extracted with an equal volume of water-saturated phenol containing 10% chloroform. Phases were separated by centrifugation, and the nucleic acid in the aqueous phase was precipitated with 2-propanol (50%). The gp41 region of interest was amplified with the GeneAmp RNA PCR kit (Perkin–Elmer/Cetus). The primer used in the reverse transcriptase reaction was the oligodeoxynucleotide 5′-CAUCAUCAUCAUCCT-GCCTAACTCTATTCACT-3′, where nucleotide 13 represents nucleotide pair 2096 of the *env* gene consensus, and 32 represents 2077 (19). The second primer used was 5′-CUACUACUACUACTGTGCCTTGGAATGCTAGT-3′, where nucleotide 13 represents nucleotide pair 1787 and 32 represents 1806. The PCR conditions were an initial 2 min at 94°C followed by 35 cycles of 1.5 min at 94°C, 1.5 min at 55°C, and 1.5 min at 72°C with a final 8.5-min incubation at 72°C. The PCR products were cloned by reacting with uracil DNA glycosylase while annealing to the pAMP vector plasmid of the CloneAMP System (Life Technologies, Grand Island, NY). Competent *Escherichia coli* DH5α cells were transformed with the annealed vector. Plasmid DNA was purified with the Magic MiniPrep DNA purification system (Promega). Inserts were verified by *Bam*HI/*Eco*RI digestion followed by electrophoresis in 1.5% agarose gels. Positive clones were sequenced by using the SP6 promoter primer (Life Technologies) in a modification of the United States Biochemical Sequenase version 2.0 protocol using 35-Sequetide (DuPont/NEN) and electrophoresed in 6% sequencing gels at 70 W for ≈2 hr. The gels were dried and exposed to x-ray film overnight at −70°C.

Table 1. Neutralization of T-lymphoid-cell-adapted HIV-1 variants

HIV-1 isolate	Neutralization end point: mAb 2F5, μg/ml	Neutralization end point: Serum 498, dilution
IIIB	6.25	1:320
AL-1	0.78	1:640
MN	0.19	1:160
WMJ-2	0.39	1:160
DU 6587-5	0.19	1:20

Antibody 2F5 Selection of a Phage Library Expressing Random Amino Acid Sequences. The recombinant phage library and the screening and PCR sequencing techniques were essentially the same as those constructed and used previously, with additional rounds of replication–expansion and reselection for selected phage as noted below (20). In brief, a phage library with a complexity of $\approx 9 \times 10^7$ recombinant phages was constructed in the fUSE5 vector (21). The phages display a random 15-mer sequence on the amino terminus of the gene III product. Antibody 2F5 was bound to polystyrene beads and the beads were incubated with $\approx 10^{12}$ phages of this library. Specifically selected phages were eluted and used to infect *E. coli* K91K cells and phage stocks were expanded. The selected eluted–expanded phage were used in two additional rounds of antibody selection. After the

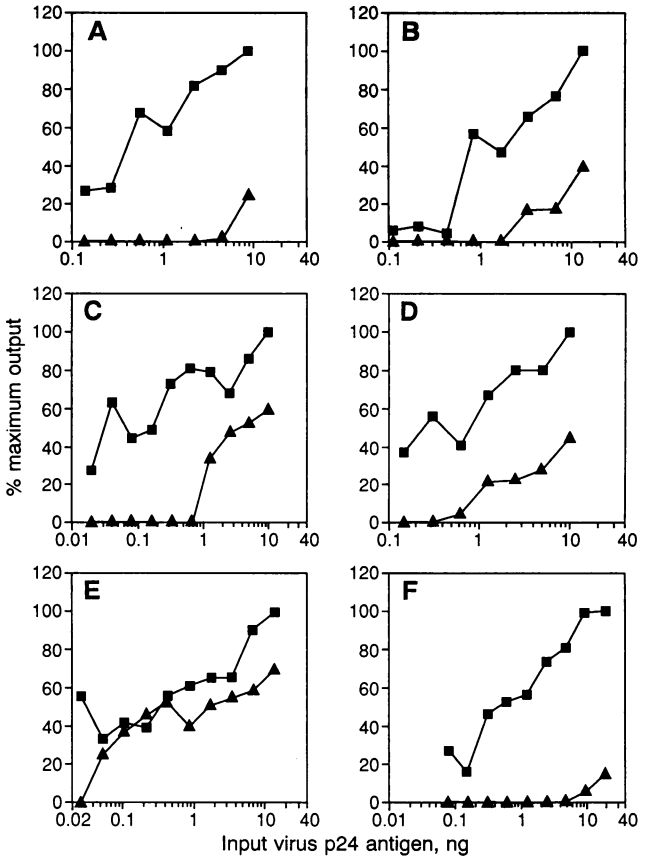


FIG. 1. Neutralization of primary HIV-1 isolates by mAb 2F5 in infectivity reduction assays. Graphs represent virus yield in the absence (■) or in the continuous presence of mAb (100 μg/ml) (▲). Isolates tested were MR452, 100% = 383 ng/ml (*A*); UAB422, 100% = 264 ng/ml (*B*); UAB112, 100% = 210 ng/ml (*C*); UAB431, 100% = 215 ng/ml (*D*); UAB430, 100% = 291 ng/ml (*E*); and 060, 100% = 615 ng/ml (*F*).

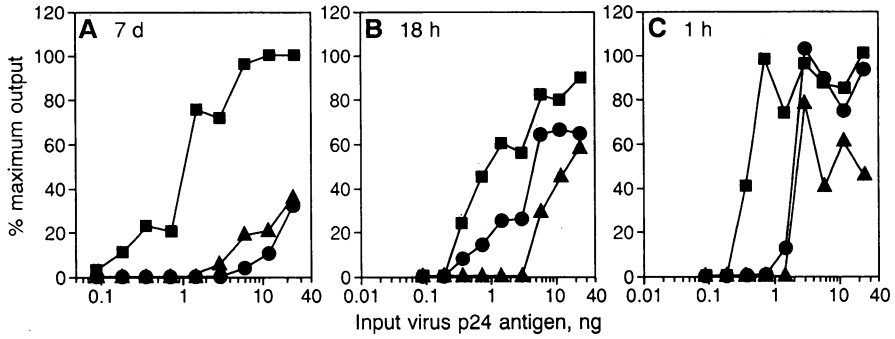


FIG. 2. Neutralizing activities of mAb 2F5 and human serum 498 against primary isolate MR452. The mAb and the serum were used at 100 μg/ml and 1:50, respectively. Antibody and serum were present throughout the incubation period of the infectivity reduction assay (*A*) or were removed 18 hr (*B*) and 1 hr (*C*) after infection. Graphs represent virus yield in the absence of antibody (■), after exposure to antibody 2F5 (▲), or after exposure to human serum 498 (●). Maximum output (100%) was 585 ng/ml (*A*), 486 ng/ml (*B*), and 416 ng/ml (*C*).

final round, colonies were lifted onto nitrocellulose and antibody 2F5 again was used as an immunological probe to confirm the selection of individual clonal colonies. The sequence of each insert was obtained by asymmetric PCR sequencing (20).

Antibody Affinity Measurement. The affinity of antibody 2F5 for the gp41 peptide ELLELDKWASLWNC (custom synthesis by Multiple Peptide Systems (San Diego) or recombinant baculovirus-produced gp41 (American Biotechnologies, Cambridge, MA) was determined by use of the BIAcore system (Pharmacia Biosensor) (22). The gp41 peptide was dissolved in 10% dimethyl sulfoxide and then diluted to 500 μg/ml with 0.01 M acetate buffer (pH 4.0). The peptide was covalently immobilized on a carboxylated dextran/gold matrix through the primary amino group by *N*-ethyl-*N'*-(dimethyl aminopropyl)carbodimide/*N*-hydroxysuccinimide chemistry (23). gp41, reconstituted in water from a lyophilized preparation, also was immobilized through the primary amino group. The antibody in 10 mM Hepes, pH 7.4/150 mM NaCl/3.4 mM EDTA/0.05% surfactant 20 (Pharmacia) was added in a flow passing over the immobilized peptide. Changes in the refractive index, in the form of the surface plasmon resonance caused by the binding and subsequent dissociation of the antibody, were monitored continuously. The kinetic rate and equilibrium constants were calculated from the collected data.

RESULTS

Neutralization of T-Lymphoid-Cell-Line-Adapted HIV-1 Variants. The virus-neutralization characteristics of mAb 2F5 were determined with laboratory-adapted HIV-1 variants. These virus strains express divergent gp120 V3 domain sequences but are generally conserved at the gp41 2F5-specific binding site (19). In all cases, 2F5 mediated ≥90% virus neutralization at antibody concentrations ranging from 0.19 to 6.25 μg/ml (Table 1). The higher concentration of 2F5 necessary at the end point for strain IIIB may reflect the rapid kinetics of replication and higher output of IIIB in MT-4 cells. This observation is comparable to the action of neutralizing mAb directed to gp120 targets (24). Human serum 498, assayed in parallel, neutralized these viruses at levels consistent with previously obtained results (18).

Class	Number	Name	Sequence
A	1	F16	D K **D** **K** **W** A S L D A R T Q M W
	2	F17	E N A I **D** **K** **W** A T L Y W A Y G
	3	F14	A F M T V **D** **K** **W** A M F W P T Q
	4	F15	H M S P F **D** **K** **W** E F I T A R R
	5	F22	S L F S E **D** **K** **W** S Y L H S N A
	6	F12	M S H Y D I **D** **K** **W** G G M T S Q
	7	F11	T P D G T L F F **D** **K** **W** S L F R
	8	F26	P K M G S L H M N **D** **K** **W** V S V
	group consensus		_ _ _ _ S L _ D F **D** **K** **W** A S L T S R _ _ _ _
B	9	F28	D D **L** **D** R **W** G R T D W N F F L
	10	F6	E D V **L** **D** R **W** V L L D H W D S
	11	F4	G Q T A A D **L** **D** K **W** Y V G G D
	12	F9	x P L P S D **L** **D** K **W** E F L N R
	13	F13	V V N S L P **L** **D** R **W** A Y S Y E
	group consensus		_ _ _ _ D D **L** **D** R **W** _ _ L D _ _ _ _ _
C	14	F27	M K **E** D D K **W** V P V K G R K F
	15	F19	M L **E** G D K **W** S D I G M V F V
	16	F1	R G R A D **E** P D R **W** A L M F D
	17	F25	T A N S H W L **E** Y D R **W** S E V
	group consensus		_ _ _ _ M L **E** _ D**K/R** **W** S _ _ _ _ _ _ _
D	18	F20	**E** **L** **D** R **W** A E L E M R G H L N
	19	F24	**E** **L** **D** K **W** G W M A S H E A H I
	20	F23	V F **E** **L** **D** K **W** S G R D E E W A
	group consensus		_ _ **E** **L** **D** K **W** _ _ _ _ _ _ _ _ A _ _

FIG. 3. Amino acid sequences selected by phage library screening with antibody 2F5.

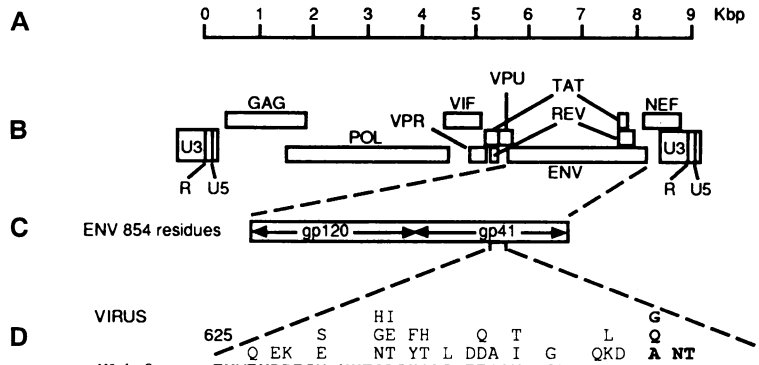
Neutralization of Primary HIV-1 Isolates. Six different isolates were tested by the infectivity reduction assay (Fig. 1). With a constant antibody concentration of 100 μg/ml throughout the assays, five of the six isolates appeared to be sensitive to 2F5. Susceptibility to neutralization varied. Isolate 060 (Fig. 1*F*) was the most susceptible whereas isolate UAB431 (Fig. 1*D*) was comparatively less so. Isolates MR452, UAB422, and UAB112 (Fig. 1 *A–C*) typified the susceptibility of primary HIV-1 isolates with 100% neutralization in the virus input range of 0.6–1.2 ng of p24 antigen. Only isolate UAB430 (Fig. 1*E*) was resistant to neutralization.

The virus-neutralizing characteristics of the mAb were further assessed by using isolate MR452. The antibody and human serum 498 were tested for their ability to neutralize the virus upon continuous virus–antibody exposure or following short exposure periods of 1 and 18 hr postinfection (Fig. 2). The serum was somewhat less effective when tested under the short exposure conditions, especially when removed at 18 hr. Antibody 2F5 retained significant neutralizing activity even when incubated with the virus for only 1 hr after infection.

Identification of the Antibody Binding Site. The gp41 binding domain for antibody 2F5 was previously reported on the basis of synthetic-peptide binding analyses (16). We performed experiments to confirm and extend these observations by using the antibody to select recombinant clones from a phage library expressing random 15-amino acid sequences (Fig. 3). None of the clones expressed the exact viral binding sequence. However, after classification of the sequences into four similarity groups and derivation of a consensus sequence for each group, the original binding-site designation could be confirmed. This sequence, ELDKW, was present in the consensus for group D. Groups A–C were closely related and all three expressed a core recognition sequence of D(K/R)W.

Antibody Affinity. By use of the BIAcore system and a peptide representative of the gp41 sequence and gp41, we were able to calculate rate and equilibrium constants for antibody 2F5. The association rate constant (k_a) of 2F5 for this gp41 peptide, measurable with antibody concentrations from 6.25×10^{-8} to 1×10^{-6} M, was 1.23×10^5 $M^{-1} \cdot s^{-1}$. The dissociation rate constant (k_d) of 2F5, measured at an antibody concentration of 1×10^{-6} M, at which the binding of the antibody was near equilibrium, was 9.43×10^{-5} s^{-1}. Thus, the half-time ($t_{1/2}$) for dissociation was calculated to be 122 min and the equilibrium association constant (K_a) was 1.3×10^9 M^{-1}. The k_a of 2F5 for gp41 was 1.8×10^4 $M^{-1} \cdot s^{-1}$ and the k_d was 7.4×10^{-5} s^{-1}. For gp41–2F5 the $t_{1/2}$ was 156 min and the K_a was 2.4×10^8 M^{-1}.

Correlation of Antibody 2F5 Neutralizing Activity with Expression of the Binding Site. To determine whether the results of the primary-isolate virus neutralization assays correlated with the presence of the binding site, we sequenced the appropriate region of the viral *env* gene from the six isolates used in our study. Sequences were obtained from each of the virus stocks as well as from plasma viral RNA



E

A1-1	.....EREID NYTSLIYTLI EESQNQQEKN EQELL**ELDKWA** SLWNWF	6/6
MR452	TWMEWEREID NYTNLIYNLI EESQNQQEKN ELELL**ELDKWA** NLWNWF	4/6
	---G------ ---S---T-- ---------- ---------- ------	1/6
	---------- ---S---T-- ---------- ---------- ------	1/6
UAB422 (virus)	TWMQWEKEID NYTGYIYNLL EESQNQQEKN EQDLL**ELDKWA** NLWNWF	4/6
	---------- -------T-- ---------- -----**A**----- ------	1/6
	---------- ---------- ---------- -----**A**----- ------	1/6
UAB422 (plasma)	....LI EESQNQQEKN EQDLL**ALDKWA** NLWNWF	5/5
UAB431 (virus)	TWMEWEREID NYTSLIYTLI EESQTQQEKN EKELL**ELDKWA** SLWNWF	3/6
	---------- ---------- ---------- ---------- N-----	1/6
	---------- ----I----L -K--N----- ----------**T** N-----	1/6
	---------- ---G-----L -K--N----- -Q--------- ------	1/6
UAB431 (plasma)	....SIIYTLL EKSQNQQEKN EKELL**ELDKWA** SLWNWF	2/6
	....G------ ---------- ---------- ------	1/6
	....GL----I -E--T----- ---------- ------	1/6
	....GL----- ---------- -Q--------- ------	2/6
UAB112 (virus)	TWMEWEKEID NYTGLIYTLL EESQIQQEKN EQELL**ELDKWA** SLWNWF	5/6
	---------N ---------- ---------- ---------- ------	1/6
UAB112 (plasma)	....LIYTLL EESQIQQEKN EQELL**ELDKWA** SLWNWF	7/8
	....------ ----V----- ---------- ------	1/8
UAB430 (virus)	........ID NYTSTIYTLI TESQTQQEKN ELELL**KLDNWA** SLWSWF	5/5
UAB430 (plasma)	.......EID NYTSTIYTLI TESQTQQEKN ELELL**KLDNWA** SLWSWF	3/3
060sup (virus)	....GIIYNLI ETSQNQQEKN ELELL**ELDKWA** SLWNWF	6/7
	....------- ----K----- ---------- ------	1/7

FIG. 4. Amino acid sequences of the region encompassing HIV-1 envelope glycoprotein residues 625–671. The sequences were derived from DNA sequence analysis of the *env* genes from the primary viral isolates used in this study. (*A*) Provirus map coordinates. (*B*) HIV-1 provirus genomic organization. (*C*) HIV-1 *env* region. (*D*) Sequence of the prototypic NL4-3 provirus. (*E*) Sequences of primary virus isolate stocks and associated plasma viral RNA (see text). At right is indicated for each sequence the number of clones expressing the sequence relative to the total number of clones analyzed. Dots indicate that no sequence was generated; dashes indicate that the residues at the designated position are identical to the first sequence listed.

obtained from the infected patients that yielded the isolates (Fig. 4). Seventy-seven new clonal sequences were derived. Clones from antibody-sensitive isolates expressed the EL-DKWA sequence with occasional minor variations. Plasma and viral stock sequences correlated with the exception of isolate UAB422. Nonetheless, in this case, the viral stock predominantly expressed the antibody binding sequence. Antibody-resistant isolate UAB430 did not contain the EL-DKWA sequence but instead expressed a divergent sequence that was notable for the substitution of an asparagine residue for lysine in the center of the binding site, KLDNWA. This is most likely the cause of the neutralization resistance exhibited by this isolate. The phage-library selection studies (see above) suggest that antibody 2F5 binding may require a lysine or arginine residue at this central position.

DISCUSSION

We used a series of HIV-1 neutralization experiments to characterize a newly described anti-gp41 human mAb. Antibody 2F5 neutralized a panel of divergent T-lymphoid-cell-adapted variants of the virus. These variants exhibit diverse gp120 V3 domain sequence. Cross neutralization of the variants by anti-V3 antibodies is usually restricted, with one exception of mAb 447-52D (24). Antibody 2F5 also effectively neutralized a panel of primary HIV-1 isolates. Neutralization was associated with expression of the antibody's binding site. We also confirmed the site's identity.

The antibody's activity against the primary isolates is particularly noteworthy. Such isolates are typically resistant to the neutralizing effects of anti-gp120 antibodies, both V3 domain- and CD4 binding site-directed (unpublished observations). In this regard, the activity of antibody 2F5 is unique since it mediated a 100% reduction of virus infectivity even at relatively high virus input levels. To our knowledge, such a degree of primary isolate neutralization has not been previously reported. However, a series of primary-isolate neutralization studies with anti-gp120 V3 region mAb 447-52D have shown that the two antibodies are approximately equal in action (A.J.C., J.A.K., L.J.B., and E.A.E., unpublished work). Further, a titration of 447-52D indicates that the proportion of input neutralized is a function of the concentration of mAb. In addition, antibody 2F5 was capable of effectively neutralizing the virus after a short exposure, suggesting that the antibody binds to its target site with high avidity. This antiviral observation is in agreement with, and the tentative conclusion is supported by, the extremely long $t_{1/2}$ of 2F5, 122 min for the gp41 peptide containing the binding site and 156 min for gp41 itself. The biological significance of these characteristics is unproven, but can be assessed by chimpanzee passive-protection experiments.

It appears that only 47–56% of HIV-1-infected humans have any detectable antibody response to the region of gp41 that contains this epitope (25). Further, this antibody appears to have virus-neutralization characteristics *in vitro*, and that neutralization ability can be abrogated by preincubation with peptide containing the 2F5 epitope. A possible protective role of antibody directed to this region of gp41 was suggested from studies where sera from non-HIV-1-transmitting mothers bound gp41 peptides to this region more frequently than sera from a group of HIV-1-transmitting mothers (26). The effect of mAb 2F5 on primary isolates reported here further strengthens the hypothesis that anti-gp41 antibody directed to this region may have a protective role.

Antibody 2F5's binding domain on gp41 is significantly represented among reported HIV-1 envelope glycoprotein sequences from geographic variants. Of the sequences available in the current Los Alamos database (19), 76% express the binding-site LDKW core. The domain is present among four of the five viral subtypes (clades) that have been described on the basis of sequence divergence (19). Given the additional sequences of this study (Fig. 3 and unpublished data) the binding-site LDKW core is present in 83% of the total sequences. These observations suggest that an immunogen capable of eliciting a 2F5-like antibody response could be a beneficial part of an effective HIV-1 vaccine. The development of such an immunogen should be considered.

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