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Biologic and Genetic Characterization of a Panel of 60 Human Immunodeficiency Virus Type 1 Isolates, Representing Clades A, B, C, D, CRF01_AE, and CRF02_AG, for the Development and Assessment of Candidate Vaccines

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A critical priority for human immunodeficiency virus type 1 (HIV-1) vaccine development is standardization of reagents and assays for evaluation of immune responses elicited by candidate vaccines. To provide a panel of viral reagents from multiple vaccine trial sites, 60 international HIV-1 isolates were expanded in peripheral blood mononuclear cells and characterized both genetically and biologically. Ten isolates each from clades A, B, C, and D and 10 isolates each from CRF01_AE and CRF02_AG were prepared from individuals whose HIV-1 infection was evaluated by complete genome sequencing. The main criterion for selection was that the candidate isolate was pure clade or pure circulating recombinant. After expansion in culture, the complete envelope (gp160) of each isolate was verified by sequencing. The 50% tissue culture infectious dose and p24 antigen concentration for each viral stock were determined; no correlation between these two biologic parameters was found. Syncytium formation in MT-2 cells and CCR5 or CXCR4 coreceptor usage were determined for all isolates. Isolates were also screened for neutralization by soluble CD4, a cocktail of monoclonal antibodies, and a pool of HIV-1-positive patient sera. The panel consists of 49 nonsyncytium-inducing isolates that use CCR5 as a major coreceptor and 11 syncytium-inducing isolates that use only CXCR4 or both coreceptors. Neutralization profiles suggest that the panel contains both neutralization-sensitive and -resistant isolates. This collection of HIV-1 isolates represents the six major globally prevalent strains, is exceptionally large and well characterized, and provides an important resource for standardization of immunogenicity assessment in HIV-1 vaccine trials.

One of the obstacles in the assessment of candidate human immunodeficiency virus type 1 (HIV-1) vaccines is the array of challenges presented by the laboratory assay validation process and the paucity of reagents available for standardization of these assays internationally. Multiple efforts to address these issues have been initiated and coordinated through the World Health Organization (WHO), the Gates Vaccine Enterprise, the Partnership for AIDS Vaccine Evaluation laboratory network, International AIDS Vaccine Initiative, and the HIV Vaccine Trials Network (HVTN) (D. Montifiore, HVTN Full Group Meet., Bethesda, MD, 2 to 5 May 2004). An important objective that has been highlighted is the development of a panel of well-characterized viral isolates (45). It is generally believed by many HIV vaccine researchers that the induction of both cellular and humoral immunity may be required of a successful vaccine candidate; the most obvious and immediate application for a viral isolate panel would be its use in assays to reproducibly assess HIV-1-neutralizing antibodies against multiple clades. Such a panel might then be applied to the comparison of neutralization assays performed in different laboratories, as well as, ultimately, the comparison of the potency of different vaccine products. Neutralizing antibodies have been shown to be effective at protecting macaques from infection of simian immunodeficiency virus (SIV) or HIV-1/SIV chimeric virus through passive transfer (3, 38, 42, 49, 62). It is therefore likely that the production of broadly cross-neutralizing antibodies will contribute to vaccine efficacy and will be a desirable property of candidate vaccines, warranting studies of not only the homologous vaccine strain, but also intra- and interclade viral neutralization.

Eliciting strongly neutralizing antibodies is currently an imposing challenge. It is presumed that neutralizing antibodies are directed against the envelope spike, which consists of a trimer of gp120 molecules bound to a trimer of membrane-spanning gp41 molecules (58). The structure of gp120 has multiple properties that may combine to provide resistance or sensitivity to neutralization. One property is the presence of five variable loop regions (V1 to V5) that are altered under selection pressure. These nonrandom mutations (7–9) may allow the virus to rapidly evade immune detection. However, increased variability does not necessarily lead to protection for

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the virus, since genetic diversity of envelope was found not to correlate with increased neutralization resistance (20).

One of the most informative means of characterizing a viral isolate is complete genome sequencing, which provides discrimination between those infected with a pure clade or established circulating recombinant form (CRF) or with a unique recombinant strain and also provides the genetic clade of the envelope, which may pertain to selection of isolates for specific assays. Previously, several attempts have been made to determine whether clade plays a role in functional antibody responses. Clade specificity and neutralization serotypes have been suggested in studies using subtype B and CRF01_AE (39, 41) and in a recent report by Binley et al. (5) using multiple clades and several monoclonal antibodies (MAbs). However, in a majority of studies of cross-clade-neutralizing antibody, the data indicated that genotype appeared to be poorly related to immunologic serotype (6, 31–33, 46, 50, 51, 68, 69). In most of these studies, the viral strains in the reagent panels were not completely sequenced, and the possibility exists that clade-specific reactivities were not identified, due to the inadvertent inclusion of genetic recombinants in the virus panels. In addition, the biological properties of HIV isolates, to include coreceptor usage and syncytium-inducing phenotypes, are an important aspect of virus characterization that were often not included in previous cross-clade neutralization studies. Transmission of virus from donor to recipient tends to result in the establishment of a CCR5-utilizing (R5) virus despite the introduction, in some cases, of a CXCR4-utilizing (X4) virus (13, 16, 55). Interestingly, in mother-to-infant transmission, the early emergence of an X4 virus in children correlated with mothers who harbored an X4 viral infection (13). Thus, it may be important for a panel of isolates whose purpose is to assess neutralization to include X4 viruses, since it is likely that X4 isolates are also transmitted upon initial infection and may have some effect on disease outcome.

Multiple HIV-1 vaccines are currently being tested, and several candidates are in the pipeline for phase I and II trials (http://www.hvtn.org/science/trials.html). The development of a standard panel of HIV-1 primary isolates to test immunologic responses and particularly to measure cross-clade-neutralizing antibodies will be paramount as investigators attempt to compare different vaccines and assess incremental improvements in vaccine performance. A globally relevant panel will also provide an important tool for assessing products of multiple clades being tested at international sites and for preparation to conduct phase III trials. In this study, we report complete genetic and biological characterization of a panel of 60 full-length (FL) sequenced HIV-1 isolates from 15 countries, including R5 and X4 viruses, representing clades A through D, CRF01_AE, and CRF02_AG for use in HIV vaccine development and in assay standardization.

MATERIALS AND METHODS

Isolation and expansion of international HIV-1 isolates. HIV-1 isolates were obtained from patient peripheral blood mononuclear cells (PBMC) (from the indicated countries) by coculture with CD8-depleted donor PBMC for up to 1 month. Donor PBMC were obtained from leukophoresis of HIV-negative donors; PBMC were isolated by Ficoll-Hypaque density gradient centrifugation and cryopreserved. Donor PBMC were thawed, CD8 depleted with Dynabeads M-450 CD8 (per the manufacturer's instructions, Dynal Biotech, NY), and stimulated with 1 μg/ml phytohemagglutinin (PHA) (Difco, Detroit, MI) in interleukin-2 (IL-2) medium (cRPMI) for 16 to 18 h at 37°C. cRPMI consists of RPMI 1640 (Quality Biologics, Gaithersburg, MD) with 1% penicillin-streptomycin (Quality Biologics), L-glutamine (Quality Biologics), and 15% fetal calf serum (FCS) (Gemini Bioproducts, Woodland, CA) containing 10% recombinant IL-2 (Roche, Indianapolis, IN). The cells were then washed with fresh IL-2 medium and cultured for 3 to 4 days before use. Cocultures were assessed two to three times each week for p24 levels (measured by p24 antigen capture; Beckman-Coulter, Miami, FL), fed fresh medium three times a week, and fed fresh donor cells one to two times a week. Viral supernatant was harvested from cultures when p24 values exceeded 50 ng/ml and was stored in 0.5- to 1-ml aliquots in a liquid nitrogen freezer. Titers of the viral stocks were then determined to obtain the 50% tissue culture infectious dose ($TCID_{50}$).

When required, viral stocks were expanded. Isolates were expanded by suspension of 10 million CD8-depleted PHA-stimulated PBMC in 1 ml of viral supernatant and incubation at 37°C for 1 h. Cells were then suspended in 10 ml of IL-2 medium and incubated for 16 to 18 h at 37°C. The next day, the cells were washed with fresh IL-2 medium and cultured for 5 to 10 days. The expansion cultures were harvested at peak virus production as measured by p24 antigen production, frozen in aliquots, and stored in a liquid nitrogen freezer for later use.

Titration of HIV-1 isolates. Titers of the viral stocks were determined in fourfold dilutions, using four replicate wells per virus dilution. Viral dilutions were added in 25-μl aliquots to 25 μl of cRPMI and incubated for 30 min at 37°C in a deep-well 96-well plate. A 50-μl aliquot containing 1.5×10^5 cells was then added to each well, and plates were incubated for 16 to 18 h at 37°C. The following day, the titration plates were washed twice with 400 μl of wash medium (same as IL-2 medium, but with 2% FCS instead of 15% FCS and without IL-2), resuspended in 200 μl of IL-2 medium, and transferred to round-bottom 96-well plates. On days 4 and 6, 100 μl of supernatant was removed and replaced by 100 μl of fresh IL-2 medium. On day 8, the supernatant was removed for p24 antigen capture. The $TCID_{50}$ was calculated by the Spearman-Karber method.

DNA sequencing. PCR amplification, complete genome sequencing, and envelope sequencing of HIV-1 strains was performed as previously described (43, 59, 61). The GenBank accession numbers for the 60 (21 new) complete genomes and for the 41 new envelope sequences from the virus cultures reported here are all listed (see Fig. 2; previously published complete genome sequences are indicated in italics). The newly reported complete genome sequences in this study were from either patient PBMC or PBMC harvested at the termination of the viral culture, usually between days 14 to 28. All envelope sequences were obtained from PBMC from the stock viral culture.

Genetic analysis. Complete genome sequences or gp160 sequences were aligned with reference sequences of the important clades and CRF. The alignment was gap stripped and resampled with the bootstrap (100 iterations). Distance matrices generated using the Kimura two-parameter method were used to construct phylogenetic trees by neighbor joining. Bootstrap values for relevant nodes were reported on a representative tree.

Translated protein sequences from the gp160 genes were generated and aligned, and a pairwise distance matrix was generated by the DAYHOFF PAM substitution method. Pairwise protein distances were divided into intervals and plotted against the number of comparisons in each interval. Potential N-glycosylation sites were evaluated with *N*-glycosyte (http://www.lanl.gov). Means and standard deviation (SD) of the number of sites were computed for isolates within each clade or CRF. The V4 region of the envelope protein was evaluated for its cysteine-to-cysteine length for each strain. Loop lengths within each clade or CRF were compared by means and SD. Differences between the clades and/or CRFs were statistically analyzed by the Kruskal-Wallis test and further characterized by multiple comparison analysis at the 0.05 alpha level.

Syncytial characterization of HIV-1 isolates. Isolates were biologically characterized by the MT-2 syncytium-forming assay. In duplicate wells, 100 μl containing 200 $TCID_{50}$ of each isolate was incubated with 5×10^4 MT-2 cells in a final volume of 200 μl. The cells were examined microscopically for syncytia every 2 to 3 days for 30 days, and isolates were identified as syncytium inducing (SI) if three or more syncytia were observed in each of the duplicate wells at the same time point and as non-SI (NSI) if no syncytia were observed. Uninfected cells and cells exposed to HIV-1 Ba-L were used as negative controls; HIV-1 III_B was used as a positive control for syncytium formation.

Coreceptor determination by the GHOST cell assay. The GHOST cell infection assay was used for determining coreceptor usage. The GHOST assay was performed by incubating 300 μl containing 300 $TCID_{50}$ of each isolate with 10^5 GHOST cells per well in 24-well plates. Parental, CXCR4-expressing, or CCR5-expressing GHOST cells were infected in the presence of 7.5 μg/ml of DEAE-dextran in duplicate wells for 4 h. The cells were washed and cultured with appropriate medium for 2 to 3 days and then harvested. The percentage of

infected cells expressing green fluorescent protein was measured by flow cytometric analysis using a FACSCalibur (Becton-Dickinson, San Jose, CA). Coreceptor usage was assigned according to which cell type the isolate infected. Viral isolates were designated as using only CXCR4 or CCR5 if the percentage of total infected cells of either the CXCR4- or CCR5-bearing cell lines was >90% after subtraction of the percent infection in parental cells. Isolates were considered to be dualtropic if the virus showed at least 10% of total infection in both cell lines. To definitively assess whether these isolates were dualtropic, they were further characterized by a receptor-binding competition assay (see below). In all GHOST cell assays, cells were mock infected as a negative control, HIV-1 Ba-L was used as an R5 positive control, HIV-1 MN was used as an X4 positive control, and murine leukemia virus was used as a control for green fluorescent protein expression.

Coreceptor characterization using receptor-binding competition. The coreceptor usage of viral isolates that appeared to be dualtropic, utilizing both CXCR4 and CCR5 by the GHOST cell assay, was further assessed using CD8-depleted PBMC and blocking of infection using either RANTES (to block CCR5) or AMD3100 (to block CXCR4). Briefly, PBMC target cells were depleted of $CD8^+$ cells with magnetic bead selection (Dynal, Inc.) according to the manufacturer's instructions. Duplicate aliquots (each, 0.1×10^6 in 50 μl) of PHA-stimulated CD8-depleted PBMC were incubated with 50 μl of RANTES stock (2 μg/ml), 50 μl of AMD3100 stock (2 μg/ml), a 50-μl combination of RANTES at 1 μg/ml and AMD3100 at 1 μg/ml, or 50 μl of cRPMI/IL-2 medium alone for 2 h. Virus (100 μl at 200 to 500 $TCID_{50}$) was then added, and cells were infected for 18 to 22 h. The next day, cells were washed with 4 ml of wash medium and resuspended in 400 μl of IL-2 medium alone (control) or 400 μl of IL-2 medium containing the respective inhibitors. At day 4, the cells were stained for intracellular p24 with the KC57-FITC MAb (Beckman-Coulter) and surface CD4-PE (Becton-Dickinson), as previously described (12). The number of infected (p24 antigen-positive) cells was enumerated by flow cytometry using a FACSCalibur, and the percent reduction of infected cells (in the presence of inhibitors) was calculated, compared to media controls.

Neutralization assay. Isolates were screened for neutralization sensitivity with three reagents at a single concentration in two or more independent experiments. Soluble CD4 (sCD4) was used at 10 μg/ml; a monoclonal cocktail was prepared using 25 μg/ml each of 2G12, 2F5, and 1gG1 b12; and a large pool of sera collected from HIV-1-positive patients from the United States (USHIV+) was used at a 1:40 dilution for the neutralization screens. Virus was prepared at a dilution that yielded ≥10 ng/ml p24 at 4 to 6 days postinfection, combined with either an equal volume (25 μl) of test reagent or IL-2 medium in triplicate, and incubated for 30 min at 37°C in 96-well deep-well plates. PHA-stimulated PBMC were then added at 1.5×10^5 cells per well. Plates were incubated overnight, washed three times with 0.4 ml of cRPMI containing 2% FCS, and transferred to round-bottom plates as was performed in the titration protocol (described above). On days 4 and 6, 100 μl of culture fluid was harvested and assayed for p24. To determine the cutoff for significant neutralization in the absence of a matched preinfection negative control serum pool, 21 HIV-1-negative sera were tested at 1:20 against six isolates (92UG_029, 85US_Ba-L, 98US_MSC5016, 99UG_A07412M1, 96TH_NI1046, and 02CM_0014BBY) in the same manner as the three neutralization reagents.

RESULTS

Collection of isolates. The primary goal of this project was to form a well-defined panel of HIV-1 isolates for distribution to laboratories interested in assay standardization and for use in assessing the performance of candidate vaccines in eliciting neutralizing antibodies. Three principle criteria were used to select isolates for inclusion in the panel. The main criterion was that the patient from whom the isolate was obtained showed a pure clade or CRF infection by full-length genome sequencing. The second criterion was that the panel was designed to include 10 isolates from each of the major clades and CRFs (clades A, B, C, and D, CRF01_AE, and CRF02_AG) that comprise the HIV-1 pandemic. This was done to ensure that the panel represents the global genetic and geographic diversity of HIV-1 infections. The third criterion was that the biological phenotype of the isolates included both SI and NSI viruses, with the majority being NSI/R5 viruses. The isolates were assembled from two sources. Eighteen were from previously established viruses, including some historic isolates from the WHO-Joint United Nations Programme on HIV/AIDS collection, and were chosen to ensure that candidate vaccine strains and viruses currently being used by other laboratories would be represented. For the sake of consistency within the panel, an abbreviated version of the WHO guidelines and previously suggested HIV-1 nomenclature (57) was adopted for the identification of isolates in this report and in the transmission of new FL and *env* sequences to the GenBank database. Using this guideline, the first two numbers indicate the year of isolation followed by a two-letter country code, which is separated from the isolate identifier by an underscore. The historically used and/or vaccine strains include US1 (90US_1), US4 (90US_4), BZ167 (89BZ_167), BX08 (92FR_BX08), BK132 (90TH_BK132), Ba-L (85US_Ba-L), MNp (94US_MNp), CM235 (90TH_CM235), CM240 (90TH_CM240), CM244 (90TH_CM244), SM145 (89SM_145), SE364 (90SE_364), IN20635-4 (94IN_20635-4), 93MW_965, 92UG_029, 93RW_024, 93UG_065, and DJ263 (91DJ_263). Isolates 98US_MSC5016 and 98US_MSC5007 were obtained from deployed personnel serving in the United States military. Although it is likely that these individuals became infected while abroad, it was not possible to definitively identify the country of origin of these viruses. Thus, these isolates were classified as originating from the patient's home country. The second source of viruses was recent isolations from cryopreserved patient plasma or PBMC from HIV-positive blood bank units or from subjects participating in ongoing natural history or cohort studies within Department of Defense (DOD) domestic or international sites. Most of these new isolates were from Cameroon, Kenya, Tanzania, Uganda, or Thailand. While many of these isolates were collected from samples dislinked from clinical data, contemporaneous CD4 T-cell counts were available for the isolates from the Thailand natural history cohort. These clinical data are as follows: 96TH_NP1538 (192 cells/μl), 96TH_NI1046 (850 cells/μl), 99TH_NI1052 (27 cells/μl), 96TH_NI1149 (453 cells/μl), 98TH_NP1251 (507 cells/μl), 97TH_NP1525 (778 cells/μl), and 97TH_NP1695 (20 cells/μl). Fig. 1 displays a map of the world with the United States-funded vaccine development sites for the DOD, Centers for Disease Control and Prevention, and the HVTN. Eighty-five percent of the isolates in the panel were derived from samples obtained from countries where vaccine trial sites are located. Thus, the panel presented in this report contains both historical and recent geographically diverse viruses from many countries where vaccine trial sites have been developed, making it a relevant collection of isolates for HIV-1 vaccine design and evaluation.

Genetic characterization of isolates. Figure 2 lists each viral strain with its clade and GenBank accession numbers assigned for the full genome sequence and for the viral isolate envelope sequence. Each strain was characterized by sequencing of the complete genome, except for a small portion of the long terminal repeat. Thirty-nine viruses were sequenced previously (2, 11, 12, 19, 21–24, 60). Twenty-one new complete genome sequences were generated for this study. Fig. 3A shows the phylogenetic analysis of the complete genome sequences, along with reference sequences of important clades and CRF. Each group of 10 sequences joined its respective clade or CRF with a bootstrap value of 100%. The strains within each clade

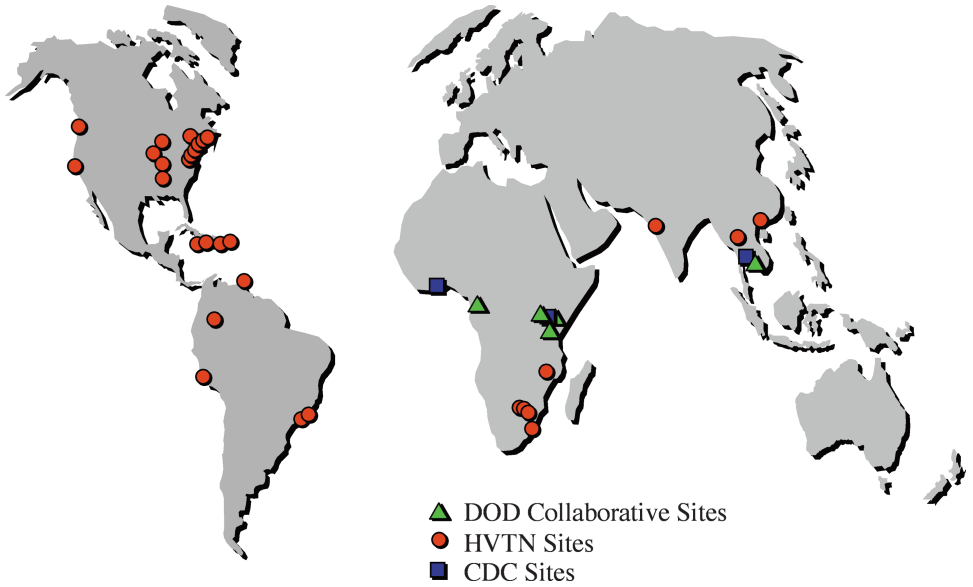


FIG. 1. Global map of U.S. government-funded HIV-1 vaccine development sites for the DOD, Centers for Disease Control and Prevention, and HVTN.

or CRF were typical of within-clade diversity and were without significant associations. Strain 00KE_KER2018 exhibited an unusually long branch within clade A, due to hypermutation. The CRF01_AE strains were more closely related than strains of other clades in this panel, reflecting the restricted diversity of CRF01_AE that remains characteristic of the HIV-1 epidemic in Thailand.

The current geographic range of each clade or CRF was to varying degrees reflected in the countries of origin of these strains. Clade A strains were from Kenya (KE), Uganda (UG), and Rwanda (RW); clade B strains were from the United States (US), France (FR), Thailand (TH), and Brazil (BR); clade C strains were from Tanzania (TZ), Senegal (SE), Somalia (SO), Malawi (MW), Ethiopia (ET), India (IN), and from a United States citizen probably infected overseas (MSC5016); and clade D strains were from Kenya (KE), Uganda (UG), and Cameroon (CM). The CRF01-AE isolates were from Thailand, and the CRF02_AG isolates were from Cameroon (CM), Djibouti (DJ), and from a United States citizen probably infected in Europe (MSC5007). With the exception of some of the historic clade B strains, all were derived from low-passage, primary PBMC coculture. None of the viral stocks used in this study were derived from cell lines.

Propagation of HIV-1 in cell culture can restrict the diversity of the quasispecies present in the original blood sample and can favor the replication of some members of the quasispecies. Thus, the portion of the viral genome relevant for neutralization studies, envelope gp160, was reamplified from the PBMC of the cultures that produced the virus stocks and resequenced. This was done for the 39 strains for which a complete genome sequence had been previously available from patient PBMC. For the 21 strains with no complete genome reference sequence, the complete genome was sequenced from the virus culture, and the envelope portion was included in the analysis shown in Fig. 3B. The mean pairwise distances of the envelope gene among the original and expanded strains, when such a comparison was possible, ranged from 0.5 to 2.5% (data not shown). Figure 3B shows the phylogenetic analysis of all 60 envelope sequences. Each envelope sequence joined its respective clade with a bootstrap value of 100%. The diversity of strains within each clade was similar to that observed with the complete genome sequences (Fig. 3A); as anticipated, strain 00KE_KER2018 was recovered in its normal, nonhypermutated form.

The genetic analysis of the virus panel established that 40 strains from clades A through D were nonrecombinant and could be assigned to their respective clades, representing diversity within that clade with high confidence. The CRF01_AE and CRF02_AG strains likewise faithfully represented their respective CRF and mirrored the diversity recognized for these CRF, currently low for CRF01_AE and higher for CRF02_AG (28, 65). The envelope sequences verify the faithful propagation of the viruses, reestablish the genetic relationships observed from the complete genome sequences, and provide an envelope sequence proximal to the virus stock that would undergo neutralization analysis.

We then examined the translated protein sequences with respect to diversity and some features that may be related to neutralization susceptibility. First, the envelope protein diversity within each subtype or CRF was determined. For example, a protein distance matrix was generated among the subtype A strains, the range of protein distances was divided into intervals, and the number of strain comparisons in each interval was calculated (Fig. 4A, top), a process repeated for each subtype or CRF. Figure 4A shows that the diversity of the protein sequences is similar for all, with the exception of the CRF01_AE strains. These isolates have envelope proteins that are more closely related to one another relative to the others.

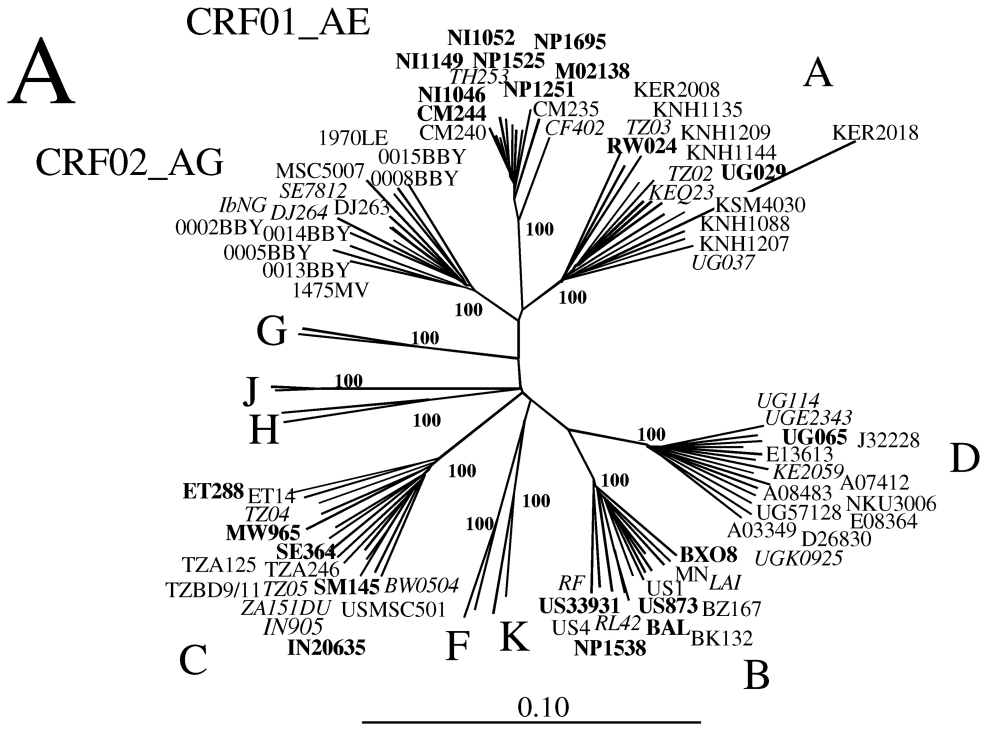
The susceptibility of HIV-1 to antibody-mediated neutralization depends in part on the number and position of N-linked carbohydrate residues. In Fig. 4B, the top panel shows an enumeration of potential N-linked glycosylation sites (NXS/T)

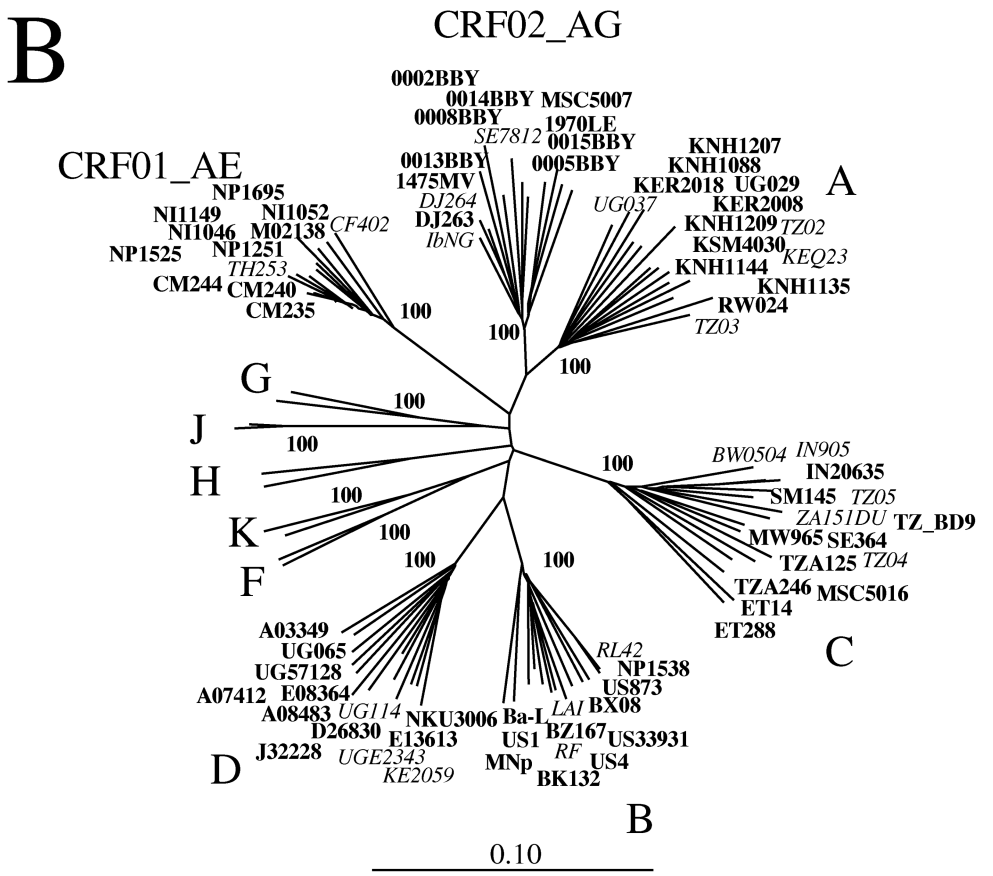
		Genbank Accession Number[a]		Viral Stock		Biological Phenotype		Neutralization Screen		
Clade/CRF	Isolate	Full-length	Env	$TCID_{50}$[b]	p24 (ng/ml)	Syncytium induction	Correceptor	sCD4	Cocktail	USHIV+
A	92UG_029	AY713407	NA	33792	113	SI	X4	100	95	19
A	93RW_024	AY713406	NA	192	176	SI	Dual	61	72	2
A	00KE_KER2008	AF457052	AY736809	6947	95	SI	Dual	2	70	0
A	00KE_KER2018	AF457057	AY736810	106	201	NSI	R5	0	99	96
A	99KE_KNH1088	AF457063	AY736811	2172	108	NSI	R5	0	86	11
A	99KE_KNH1135	AF457065	AY736814	874	187	NSI	R5	14	91	36
A	00KE_KNH1144	AF457066	AY736812	874	242	NSI	R5	18	69	46
A	00KE_KNH1207	AF457068	AY736815	905	480	NSI	R5	1	79	28
A	00KE_KNH1209	AF457069	AY736813	192	427	NSI	R5	12	81	46
A	00KE_KSM4030	AF457079	AY736816	309	227	NSI	R5	0	63	64
B	94US_33931N	AY713410	NA	1748	282	NSI	R5	55	99	60
B	90US_873	AY713412	NA	57501	515	NSI	R5	17	87	50
B	85US_Ba-L	AY713409	NA	55938	661	NSI	R5	95	100	97
B	90TH_BK132	AY173951	AY736821	1386	168	SI	X4	85	94	93
B	92FR_BX08	AY713411	NA	102828	1279	NSI	R5	5	43	44
B	89BZ_167	AY173956	AY736820	19302	394	SI	X4	89	91	93
B	84US_MNp	M17449	AY736819	155	554	SI	X4	89	84	100
B	96TH_NP1538	AY713408	NA	3920	554	NSI	R5	14	82	79
B	91US_1	AY173952	AY736817	7075	400	NSI	R5	93	100	79
B	91US_4	AY173955	AY736818	768	128	NSI	R5	88	40	72
C	94IN_20635-4	AY713414	NA	11841	300	NSI	R5	96	85	21
C	98US_MSC5016	AY444801	AY736822	27877	669	NSI	R5	75	87	86
C	93MW_965	AY713413	NA	13033	710	NSI	R5	44	88	85
C	90SE_364	AY713416	AY833059	71329	273	NSI	R5	79	53	79
C	89SM_145	AY713415	AY833060	490	328	NSI	R5	15	13	13
C	02ET_14	AY255825	AY736823	362	215	NSI	R5	14	69	72
C	02ET_288	AY713417	NA	1349	84	NSI	R5	37	34	73
C	01TZ_911	AY253322	AY736826	18380	288	NSI	R5	26	90	97
C	00TZ_A125	AY253304	AY736824	426	241	NSI	R5	6	76	99
C	00TZ_A246	AY253308	AY736825	206	67	NSI	R5	9	97	19
D	98UG_57128	AF484502	AY736829	7769	211	NSI	R5	25	97	65
D	93UG_065	AY713418	NA	309	692	SI	X4	12	25	18
D	99UG_A03349M1	AF484518	AY736834	4345	964	NSI	R5	61	86	88
D	99UG_A07412M1	AF484477	AY736828	11088	565	NSI	R5	68	73	99
D	99UG_A08483M1	AY304496	AY736827	33492	594	NSI	R5	30	98	77
D	00UG_J32228M4	AF484516	AY736831	11089	721636	NSI	R5	30	78	97
D	00UG_D26830M4	AF484486	AY736830	309	431	NSI	R5	76	95	87
D	00UG_E08364M4	AF484487	AY736832	48	95	NSI	R5	38	83	95
D	00UG_E13613M4	AF484515	AY736833	693	74	NSI	R5	74	100	93
D	00KE_NKU3006	AF457090	AY736835	23412	553	NSI	R5	18	91	94
CRF01_AE	96TH_M02138	AY713424	NA	464	125	SI	X4	10	19	0
CRF01_AE	90TH_CM235	AF259954, AF259955	AY736837	2896	138	NSI	R5	1	80	67
CRF01_AE	90TH_CM240	U54771	AY736838	288	134	NSI	R5	14	67	93
CRF01_AE	90TH_CM244	AY713425	NA	66048	95	NSI	R5	2	69	78
CRF01_AE	96TH_NI1046	AY713421	NA	724	64	NSI	R5	11	69	75
CRF01_AE	99TH_NI1052	AY713423	NA	490	57	SI	X4	0	9	10
CRF01_AE	96TH_NI1149	AY713426	NA	327	124	NSI	R5	19	74	90
CRF01_AE	98TH_NP1251	AY713422	NA	25219	143	NSI	R5	25	81	63
CRF01_AE	97TH_NP1525	AY713420	NA	1629	75	SI	Dual	22	85	21
CRF01_AE	97TH_NP1695	AY713419	NA	1448	50	SI	X4	4	31	5
CRF02_AG	98US_MSC5007	AY444808	AY736840	1024	274	NSI	R5	20	85	87
CRF02_AG	01CM_0002BBY	AY371122	AY736843	38	68	NSI	R5	7	88	98
CRF02_AG	01CM_0005BBY	AY371123	AY736844	53032	122	NSI	R5	4	64	70
CRF02_AG	01CM_0008BBY	AY371124	AY736845	46092	240	NSI	R5	8	86	71
CRF02_AG	02CM_0013BBY	AY371125	AY736846	35502	102	NSI	R5	77	0	0
CRF02_AG	02CM_0014BBY	AY371126	AY736847	29489	151	NSI	R5	30	95	83
CRF02_AG	02CM_0015BBY	AY371127	AY736848	4370	114	NSI	R5	0	85	69
CRF02_AG	01CM_1475MV	AY371138	AY736842	437	241	NSI	R5	10	98	59
CRF02_AG	02CM_1970LE	AY371128	AY736841	1024	78	NSI	R5	30	87	95
CRF02_AG	91DJ_263	AF063223	AY736839	5803	96	NSI	R5	33	96	87

FIG. 2. Genetic and biological characterization of 60 international HIV-1 isolates. The 60 viruses were either expanded from previously established stocks or newly isolated. Clade purity was assessed either by performing FL sequencing or by comparing the envelope gene to previously obtained FL sequences from primary PBMC. GenBank accession numbers are listed for each FL and envelope sequence. The p24 concentration, titer, syncytial phenotype, coreceptor usage, and neutralization profile are indicated for each isolate. For the neutralization profiles, blue indicates ≤50% neutralization, while red indicates >50% neutralization. *a*, GenBank accession numbers from previously published sources are in black, and numbers submitted for this communication are in red. *b*, $TCID_{50}$ was measured from 25 μl of virus stock (see Materials and Methods). NA, not applicable, as the isolate *env* sequence is contained within the new FL sequence, obtained from culture of the virus stock. Shaded rows indicate isolates that are being used for current or candidate viruses.

in gp160, represented as the mean ± 1 SD for each clade or CRF. The CRF01_AE strains had the fewest and clade B had the most potential sites by this analysis. To assess any statistically significant differences between clades or CRFs with respect to number of potential N-linked glycosylation sites, a Kruskal-Wallis one-way analysis of variance was performed, resulting in a *P* value of 0.042. Multiple comparison analysis of Wilcoxon rank sums revealed two overlapping groups (group 1 = CRF01_AE, C, and A; group 2 = C, D, A, CRF02_AG, and B). Therefore, CRF01_AE is significantly different from subtypes B and D and CRF02_AG, alpha level 0.05, as they are not clustered in overlapping groups.

Similarly, we examined the length of the V4 variable loop (Fig. 4B, bottom), which has been correlated with neutraliza-


A
CRF01_AE
CRF02_AG
NI1052
NP1695
NI1149
NP1525
TH253
M02138
NI1046
NP1251
CM244
CM240
CM235
CF402
KER2008
KNH1135
TZ03
KNH1209
KER2018
RW024
KNH1144
TZ02
UG029
KEQ23
KSM4030
KNH1088
KNH1207
UG037
1970LE
0015BBY
MSC5007
0008BBY
SE7812
IbNG
DJ264
DJ263
0002BBY
0014BBY
0005BBY
0013BBY
1475MV
100
G
J
H
UG114
UGE2343
UG065
J32228
E13613
KE2059
A07412
A08483
NKU3006
UG57128
E08364
A03349
D26830
D
ET288
ET14
TZ04
MW965
SE364
TZA125
TZA246
TZBD9/11
TZ05
SM145
BW0504
ZA151DU
USMSC501
IN905
IN20635
C
F
K
BXO8
UGK0925
MN
LAI
RF
US1
US33931
US873
BZ167
US4
RL42
BAL
BK132
NP1538
B
0.10


B
CRF02_AG
CRF01_AE
0002BBY
0014BBY
MSC5007
0008BBY
SE7812
1970LE
0015BBY
0013BBY
0005BBY
1475MV
DJ264
DJ263
IbNG
KNH1207
KNH1088
KER2018
UG029
A
UG037
KER2008
KNH1209
TZ02
KSM4030
KEQ23
KNH1144
KNH1135
RW024
TZ03
NP1695
NI1149
NI1052
NI1046
M02138
CF402
NP1525
NP1251
TH253
CM244
CM240
CM235
100
G
J
H
K
F
BW0504
IN905
IN20635
SM145
TZ05
ZA151DU
TZ_BD9
MW965
SE364
TZA125
TZ04
TZA246
MSC5016
ET14
ET288
C
A03349
UG065
UG57128
A07412
E08364
A08483
UG114
NKU3006
D26830
E13613
J32228
UGE2343
KE2059
D
RL42
NP1538
US873
LAI
BX08
Ba-L
US1
BZ167
US33931
MNp
RF
US4
BK132
B
0.10

tion susceptibility in some studies. There was again variation within clades, with clade C having the shortest loops and CRF02_AG having the longest. The Kruskal-Wallis analysis again identified a significant difference in mean V4 length between clades ($P = 0.008$), and further analysis by multiple comparison revealed three overlapping groups (group 1 = C, CRF01_AE, D, and B; group 2 = D, B, and CRF02_AG; group 3 = B, A, and CRF02_AG). Clades or CRFs that do not overlap groups are significantly different from each other; therefore, both clade C and CRF01_AE are significantly different from both clades A and CRF02_AG (alpha level = 0.05).

These data presented in Fig. 4B pertain only to the 60 strains represented in this virus panel and may not be representative of the totality of strains in the pandemic, but the analysis shown anticipates correction factors that may need to be applied when comparing neutralization susceptibility across clades. We found no trend in the length of V1-V2 loops or in the overall length of the V1-V4 region of the envelope among the strains examined (data not shown).

Biological characterization of isolates. To characterize the infectivity and replication properties of the viruses in this panel, the $TCID_{50}$ per 25 μl was determined for each isolate, and the stock p24 antigen concentration (ng/ml) was quantified. Figure 2 lists the biological properties of the isolates in the panel, including p24 antigen concentration, $TCID_{50}$, syncytial phenotype, and coreceptor usage. The infectious titers did not correlate with the extracellular level of p24 at the time of harvest of the virus stocks (Fig. 5). Linear regression analysis showed a very low correlation coeffient ($r^2 = 0.147$) between these two biological parameters. This phenomenon has been observed in previous studies (17, 40) and indicates that viral stock p24 antigen concentration is not always reflective of the level of infectious virus contained in the stock.

To further characterize the biology of the isolates, syncytial phenotype and coreceptor usage were determined by means of the MT-2 syncytial assay and the GHOST cell assay, respectively. These important parameters of HIV-1 biology have been used previously to characterize viral isolates and to provide an indication of the stage of disease at which the isolates were obtained (as reviewed in reference 47). The MT-2 assay determines whether a virus is SI or NSI, based on its ability to use the coreceptor CXCR4. The majority of the isolates in the panel were NSI (Fig. 2 and Fig. 6A). Among the SI isolates, there were three from clade A, three from clade B, four from CRF01_AE, and one from clade D. None of the isolates from clades C or CRF02_AG were SI.

Data from the GHOST coreceptor assay supported the MT-2 results (Fig. 2 and 6A). Isolates that were SI were found to infect CXCR4-expressing GHOST cells, while NSI isolates were found to infect CCR5-expressing GHOST cells. The isolates that were tropic for CCR5 were extremely specific for the CCR5 GHOST cells, showing a range of 97.7 to 100% entry compared to total infected GHOST cells. X4-using viruses showed a range of 92.5 to 100% entry into CXCR4 GHOST cells compared to total infected cells (data not shown). While this is quite specific for a biological assay, it is likely that the lower specificity of X4 viruses for the CXCR4 GHOST cells compared to R5 viruses for CCR5 GHOST cells is due to the fact that all GHOST cell lines have low levels of endogenous CXCR4 expression (48).

Isolates 93RW_024, 00KE_KER2008, and 97TH_NP1525 were able to infect both CXCR4- and CCR5-expressing GHOST cells, suggesting that these isolates are dualtropic (X4R5) viruses. To confirm that these isolates were able to utilize both CXCR4 and CCR5, competition assays (Fig. 6B) were performed in PBMC using RANTES and AMD3100 as coreceptor inhibitors. In addition, since the X4 viruses demonstrated some promiscuity among the three GHOST cell lines, all suspected X4 isolates were included in competition assays to confirm X4 usage. RANTES alone was ineffective at inhibiting infection in all CXCR4-utilizing isolates identified in the GHOST cell assay except for 98TH_NP1525 (Fig. 6B and data not shown). AMD3100, however, was able to strongly inhibit infection of these CXCR4-utilizing isolates, except 93RW_024 and 00KE_KER2008. Interestingly, while both 93RW_024 and 00KE_KER2008 did not show a decrease in p24 positive cells in the presence of RANTES or AMD3100 alone, both entry inhibitors caused a significant inhibition of the CD4 down-regulation that is induced by these two isolates (data not shown). This suggests that both RANTES and AMD3100 affect the normal course of infection with 93RW_024 and 00KE_KER2008. Combining RANTES and AMD3100 strongly inhibited all candidate dualtropic isolates tested, including 93RW_024 and 00KE_KER2008 (Fig. 6B). This suggests that 93RW_024 and 00KE_KER2008 do in fact use both CXCR4 and CCR5, supporting the GHOST cell infection data. Although identified as a dualtropic virus (National Institutes of Health [NIH] AIDS Research and Reference Reagent Program Catalog), our data indicate that 90BZ_167 is an X4 virus. The envelope sequence of the 90BZ_167 virus in our panel matches that of the originally submitted sequence (GenBank accession no. L22087). This virus was previously reported to use CXCR4 as a major coreceptor, CCR3 as a minor coreceptor, and CCR5 not at all (14). Our findings support those of Cecilia et al. (14) that 90BZ_167 is an SI/X4 virus, as confirmed by coreceptor competition assay (Fig. 6B).

The final biological variable that was assessed for this panel was neutralization sensitivity. To determine the neutralization profiles of isolates on the panel, each virus was tested at a single dilution with three neutralization reagents: sCD4 (10

FIG. 3. Phylogenetic relationships of HIV-1 sequences from the international panel. (A) FL sequences were previously generated for 39 of the virus isolates, and 21 were newly sequenced for this study. Preexisting (plain text) and newly obtained (boldface) sequences were aligned with reference sequences (italics) of HIV-1 subtypes and relevant CRF. Strain KER2018 is hypermutated. (B) After expansion of the 60 viruses in primary PBMC coculture, gp160 was amplified from the cellular DNA and sequenced. The new sequences (boldface) were aligned with reference sequences (italics) of HIV-1 subtypes and relevant CRF. Each alignment was gap stripped, resampled using SEQBOOT (100 iterations), and analyzed with DNADIST, NEIGHBOR, and CONSENSE. Representative trees are shown with bootstrap values recorded for important nodes. The scale represents a 10% difference.

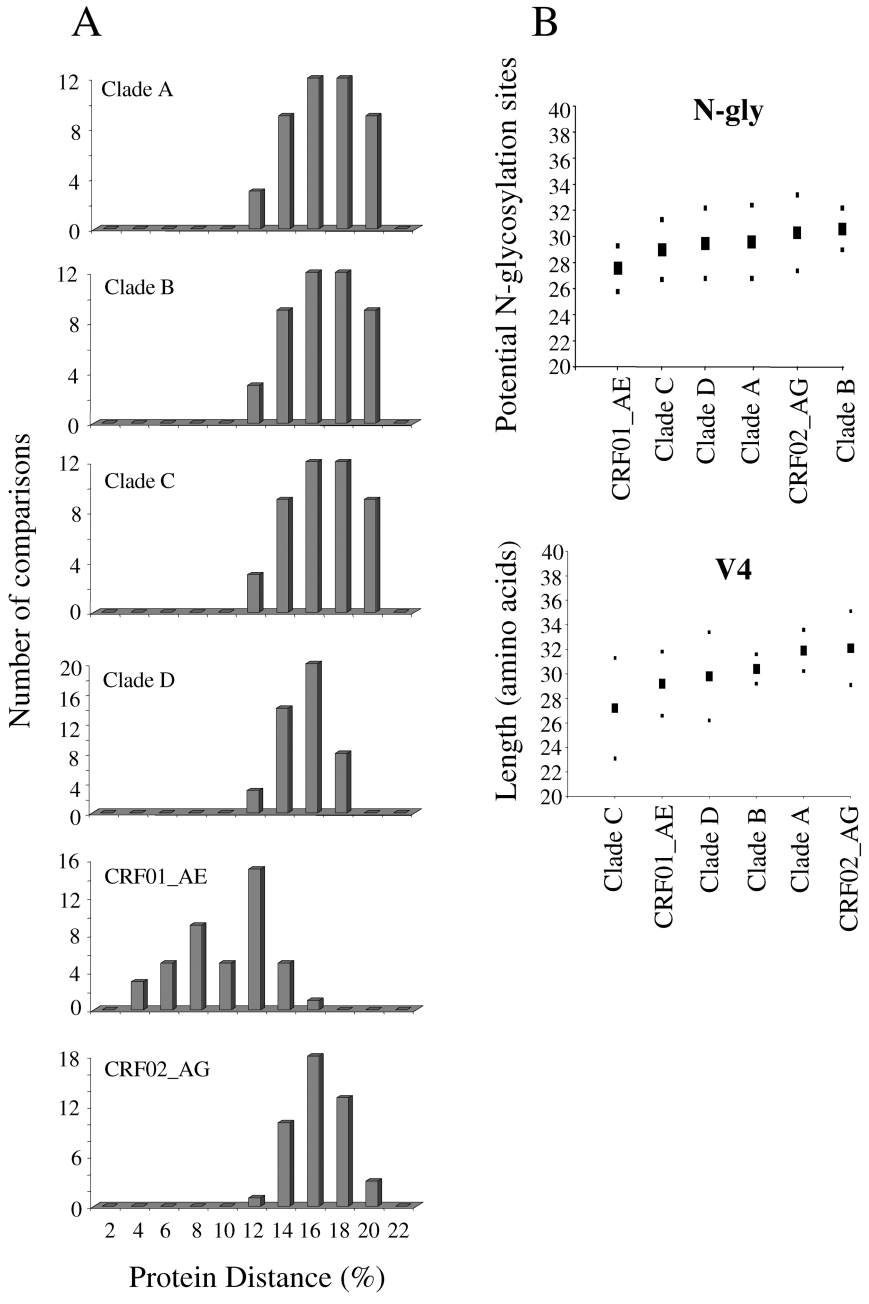


FIG. 4. Characterization of gp160 sequences. (A) Diversity of envelope sequences within subtypes and CRF represented by distributions of pair-wise protein distances. (B) Parameters implicated in neutralization resistance include average number of potential N-glycosylation sites (N-gly, top) and length of hypervariable loops (V4, bottom). Large squares indicate the means, while small squares represent the mean ± 1 standard deviation. Subtypes are arranged in order of increasing glycosylation or loop length.

μg/ml), a monoclonal cocktail containing 25 μg/ml each of 2G12, 2F5, and 1gG1 b12; and sera from the USHIV+ pool at a 1:40 dilution. At least two independent assays were performed for each isolate against each neutralization reagent, and Fig. 2 shows the mean percent neutralization. HIV-1-negative serum samples from 21 subjects were used to obtain a range of nonspecific neutralization. Six isolates, one from each clade, which appeared in preliminary experiments to be neutralization sensitive, were tested at a 1:20 dilution using the 21 negative sera. The upper limit for the 95% confidence interval for all 126 negatives was 19.22% neutralization (data not shown). However, one of the isolates, 99UG_A07412M1, was more sensitive to nonspecific inhibition than the other five isolates, with a 95% confidence interval upper limit of 49.96% neutralization (data not shown). A value of 50% was therefore chosen as the cutoff for significant neutralization. In Fig. 2, values with >50% neutralization are highlighted in red, while those with ≤50% are neutralization highlighted in blue.

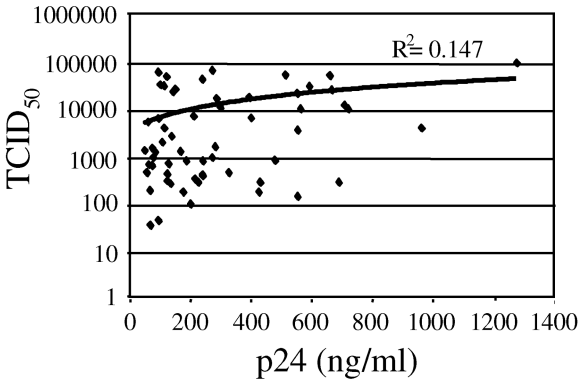


FIG. 5. HIV-1 $TCID_{50}$ and p24 concentrations are not correlated. The calculated $TCID_{50}$ was compared to viral stock p24 concentration by linear regression analysis. The trend line shows that the $TCID_{50}$ and p24 concentrations have no correlation ($r^2 = 0.147$).

Isolates that are sensitive or resistant to these three reagents may be tentatively identified by comparing the data highlighted in Fig. 2. Isolates may be considered sensitive to neutralization if they are neutralized by two or more of the reagents and resistant if neutralized by one or no reagent. For example, looking at each clade or CRF, potentially resistant viruses may be 00KE_KER2008, 92FR_BXO8, 89SM_145, 93UG_065, 96TH_M02138, and 02CM_0013BBY. In the same manner, potentially sensitive viruses may be identified, such as 00KE_KER2018, 89BZ_167, 98US_MSC5016, 00UG_E13613M4, 90TH_CM235, or 02CM_0002BBY. However, the data from these three reagents alone may not reliably predict the overall neutralization phenotypes of these viruses; these data provide only a relative indication of neutralization susceptibility. Studies using a larger panel of neutralization reagents are in progress to address this question. Of note, only 1 of 20 CRFs was neutralized by sCD4 (Fig. 2). This observation warrants further characterization. Additionally, the MAb cocktail neutralized a large portion of the isolates (51/60) and the USHIV+ serum pool neutralized more than half of the viruses in each clade or CRF, except the A clade, where 8/10 viruses were resistant to this serum pool.

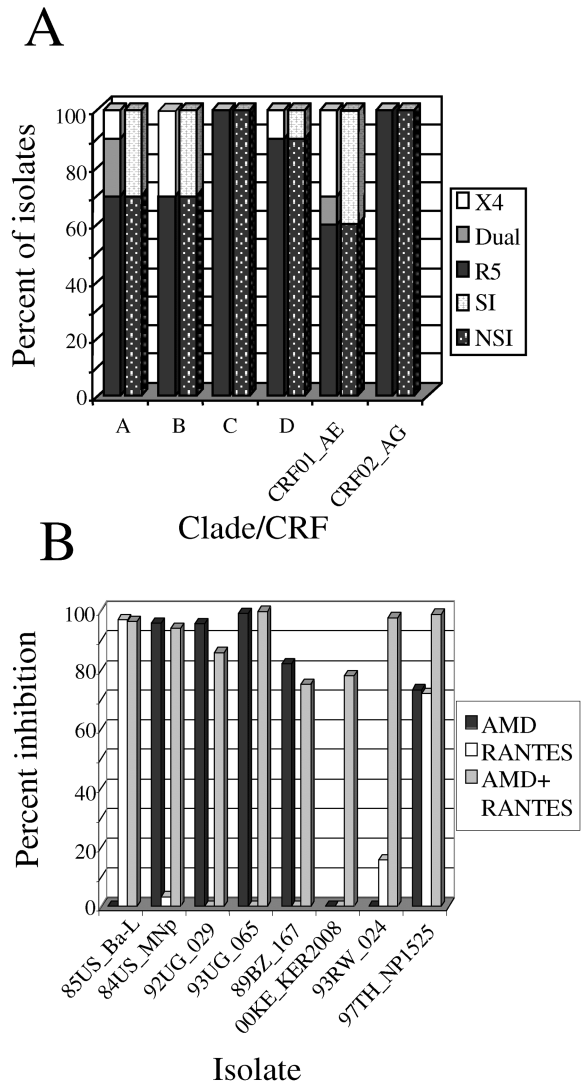


FIG. 6. Characterization of syncytium induction and coreceptor usage. (A) Viral isolate clade was compared to syncytium induction (right-hand bars) as measured by the MT-2 assay and coreceptor usage (left-hand bars) as measured by the GHOST assay (see Materials and Methods). (B) Five isolates were further characterized by performing competition assays using AMD3100 (AMD) and RANTES to determine if the isolates utilized both CCR5 and CXCR4 coreceptors.

DISCUSSION

In this study, we report the development and characterization of a large panel of HIV-1 isolates for assay standardization and for assessing neutralization in HIV vaccine research. One of the strengths of this panel is that it is exceptionally well characterized, both biologically and genetically. In addition, the isolates are from multiple countries, indicating a large geographic distribution, and include many vaccine strains and candidate vaccine strains that are representative of regions where vaccines will be developed and tested (Fig. 1). The panel also contains several isolates obtained more recently in the epidemic from sites where clades A, C, and D are prevalent (2, 19, 22, 23). Furthermore, 10 isolates from each major clade and CRF are included on the panel, thus providing the ability to assess breadth of neutralization, both within and between clades. However, as it was a challenge to obtain 10 isolates with pure complete genomes for each clade or CRF, the first available isolates identified were included on the panel. Due to the collection method for many samples (anonymous blood bank samples), the demographic and clinical data for several subjects were unknown. Additionally, isolates from the same country were not necessarily collected at the same time. These limitations identify critical parameters that should be considered in assembling viral reagents for assay standardization. Thus, while the panel will be useful for comparisons of viral phenotype and genotype, future panels should be assembled using isolates from patients with a well-characterized clinical history and for whom detailed demographic data are available to allow analysis using multiple parameters.

Within the panel, the CRF01_AE viruses display less diversity than the other groups of isolates. However, this appears to

be reflective of the current molecular epidemiology of this CRF in southeast Asia where intrapatient diversity is still lower than many countries and regions where clades and CRFs are already established (65). In addition, the CRF01_AE viruses were found to contain a lower average number of glycosylation sites with respect to other clades/CRFs within the panel. Glycosylation of the gp120 protein has been suggested as another factor involved in viral evasion of neutralizing antibodies. Glycosylation can mask neutralization epitopes, as demonstrated by structural data (71). This has also been seen in multiple studies examining the removal of glycosylation sites, which leads to increased neutralization sensitivity of the virus (26, 30, 37, 56). However, in some studies, removal of some glycosylation sites from gp120 resulted in little or no change in neutralization of HIV-1 (26, 56). Thus, it has been proposed that glycosylation may be used as a shield which results in the positioning and shifting of the glycans present on the gp120 surface to protect the envelope spike from binding of neutralizing antibodies (70). It is interesting that our statistical analyses, albeit using small sample numbers, indicated a significant difference between subtype B and CRF01_AE with respect to potential N-glycosylation sites. This is an intriguing observation in light of the fact that these two subtypes have been previously identified as immunologically (39, 41) and biologically (54) distinct. The statistical significance of the trends observed using the panel will be strengthened by increasing the number of isolates assessed for glycosylation sites and performing neutralization assays to provide an indication of the relationship between neutralization sensitivity and glycosylation patterns.

While clade C viruses showed the shortest average V4 length, the CRF01_AE viruses had the longest V4 length. In one study of clade C infections, the V1-V4 length within the envelope of viruses obtained from newly infected recipients was shown to be shorter than that of viruses found in the donors (18). The recipients' viruses were also shown to be more sensitive to neutralization by the donor's plasma (18), indicating that V1-V4 length variation may play a role in neutralization. In addition, the variable loops may also aid in neutralization resistance by masking potential neutralizing epitopes. The V1/V2 loop partially covers the CD4 binding site (34, 35, 64, 71), which would theoretically be an ideal neutralization domain. Removal of the V1/V2 loop or V2 alone has been shown to increase sensitivity to neutralization of HIV-1 and SIV (10, 25, 64). Using subunit constructs with deleted variable regions as immunogens results in redirection of the immune response (4, 29, 63). One study (4) found that animals immunized with variable loop-deleted subunits elicited antibodies able to neutralize both homologous and heterologous primary isolates more efficiently than the unmodified subunit. However, other studies found that the modified subunit was no better at eliciting neutralizing antibodies than the unmodified subunit (29, 63). This suggests that masking alone does not provide resistance to neutralization. Our data showed no trends in V1/V2 length among the clades. These observations will be further explored as the panel is characterized for cross-clade neutralization patterns.

Another obvious bias within the panel is the uneven distribution of R5, X4, and dual viruses among the clades and CRFs. A majority of the isolates obtained for the panel were NSI and utilized CCR5 as a coreceptor for viral entry. This is a desirable characteristic of the panel as it is currently thought that the early transmitted, R5 viruses are key for HIV-1 vaccine development and for use in assays to assess vaccine immunogenicity. However, some CXCR4-utilizing viruses were also included, as it has been shown that transmission of X4 viruses may have an effect on disease development (13). It has also been shown that SI viruses are more resistant to neutralization in CRF01_AE infections (54). Thus, the role of SI/X4 isolates in HIV-1 infection is unclear. No C clade or CRF02_AG isolates that utilize CXCR4 for viral entry were available for inclusion on the panel. While X4 clade C isolates have been found (1, 15, 27, 53, 66, 67), historically they are rare, and our panel reflects this. The CRF02_AG epidemic has not yet been well characterized, but some CXCR4-using CRF02_AG isolates have been reported (72). Thus, it is unclear whether the paucity of X4 isolates is reflective of the epidemiology for this CRF.

The finding that isolate p24 and titer have no correlation was not surprising. Isolates grown in culture may generate noninfectious particles, and cultures may contain virions that, although once functional, become inactivated during viral growth due to envelope shedding and/or degradation (36, 44). While it can be effective to use p24 to estimate the amount of virus present in culture supernatants, using p24 as a measurement for standardizing infectious levels of virus for neutralization assays may generate misleading results.

As can be seen by the neutralization data in Fig. 2, most isolates can be differentiated by their comparative sensitivities to sCD4, the MAb cocktail, and the USHIV+ serum pool. The percent neutralization by these three reagents provides a neutralization profile that can be used to assess whether the isolates are "relatively" resistant or sensitive to neutralization. The panel therefore contains a combination of sensitive and resistant isolates that will allow for some degree of analysis of incremental improvements in the neutralizing antibody responses elicited by candidate vaccines. The currently presented profiles are limited to three different neutralizing reagents. To assess incremental vaccine improvements, it would be advantageous for each isolate on neutralization panels to be tested against several reagents, including clade-specific individual and pooled sera, to obtain a broader profile of neutralization sensitivity for each isolate.

The panel described in this report will provide a valuable resource to HIV-1 vaccine developers; the panel will be made available for distribution through the U.S. NIH AIDS Research and Reference Reagent Program in the near future (mid-2005). The presence of an available well characterized panel of isolates will be a significant step towards improving our ability to standardize neutralization assays, as recently suggested by Moore and Burton (45). Further studies are in progress to examine the neutralization profiles of the isolates within this panel using numerous serologic reagents. In addition, the ongoing preparation of full-length gp160 clones from this entire panel will provide a useful resource for standardization between laboratories using the pseudovirus reporter assay platform (52). This panel of envelope clones may be used to complement the efforts of the Gates Vaccine Enterprise, HVTN, the NIH Division of AIDS, the NIH Vaccine Research Center, the Partnership for AIDS Vaccine Evaluation, the

International AIDS Vaccine Initiative, the WHO, and other institutes in the movement toward a validated, good laboratory practice-compliant neutralization assay using standardized viruses and controls. Currently, many assays are being developed based on pseudotyped viruses, containing a common backbone but variable primary isolate envelopes. The impact of clade in neutralization has not yet been clearly defined. One clear way to address this question is to use isolates that are full-length pure clade. Since the issue of clade specificity is unresolved for primary isolates, it is also unknown what impact this will have on pseudotype viral neutralization assays. Further studies using this characterized viral panel and envelope clones will provide important information for HIV vaccine researchers.

This is the first such panel to be supported with complete genome sequences and the first to provide a complete envelope sequence of each viral strain taken directly from the stock virus culture. These properties of the panel will aid in exploring differences in the breadth and potency of neutralizing antibodies elicited by vaccines from multiple clades and will allow for comparisons of different immunogens as candidate HIV vaccines. It is our hope that this panel will be used as one resource in the formation of broader, well-defined, clade-specific, and multiclade panels which also contain incident and recent isolates that are currently circulating in the HIV pandemic.

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The views expressed here are the private opinions of the authors and are not to be considered as official or reflecting the views of the U.S. Army of the U.S. Department of Defense. Use of trade names is for identification only and does not imply endorsement by the U.S. government.

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