JOURNAL OF VIROLOGY, Feb. 2010, p. 1439–1452 Vol. 84, No. 3
0022-538X/10/$12.00 doi:10.1128/JVI.02108-09
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Tiered Categorization of a Diverse Panel of HIV-1 Env Pseudoviruses for Assessment of Neutralizing Antibodies$^{\nabla}$

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Received 5 October 2009/Accepted 12 November 2009

The restricted neutralization breadth of vaccine-elicited antibodies is a major limitation of current human immunodeficiency virus-1 (HIV-1) candidate vaccines. In order to permit the efficient identification of vaccines with enhanced capacity for eliciting cross-reactive neutralizing antibodies (NAbs) and to assess the overall breadth and potency of vaccine-elicited NAb reactivity, we assembled a panel of 109 molecularly cloned HIV-1 Env pseudoviruses representing a broad range of genetic and geographic diversity. Viral isolates from all major circulating genetic subtypes were included, as were viruses derived shortly after transmission and during the early and chronic stages of infection. We assembled a panel of genetically diverse HIV-1-positive (HIV-1$^+$) plasma pools to assess the neutralization sensitivities of the entire virus panel. When the viruses were rank ordered according to the average sensitivity to neutralization by the HIV-1$^+$ plasmas, a continuum of average sensitivity was observed. Clustering analysis of the patterns of sensitivity defined four subgroups of viruses: those having very high (tier 1A), above-average (tier 1B), moderate (tier 2), or low (tier 3) sensitivity to antibody-mediated neutralization. We also investigated potential associations between characteristics of the viral isolates (clade, stage of infection, and source of virus) and sensitivity to NAb. In particular, higher levels of NAb activity were observed when the virus and plasma pool were matched in clade. These data provide the first systematic assessment of the overall neutralization sensitivities of a genetically and geographically diverse panel of circulating HIV-1 strains. These reference viruses can facilitate the systematic characterization of NAb responses elicited by candidate vaccine immunogens.

The development of an HIV-1 vaccine that can elicit protective humoral and cellular immunity is one of the highest priorities in the global fight against HIV/AIDS (2, 44). Data from lentiviral animal models suggest that antibodies capable of neutralizing primary strains of HIV-1 may have the capacity to prevent HIV-1 infection (1, 28, 30, 35). However, the ability to design immunogens that can elicit such broadly reactive neutralizing antibodies (NAbs) has proven to be a formidable obstacle, due in part to the extensive genetic diversity of HIV-1 and the complex escape mechanisms employed by the envelope gp120 and gp41 glycoproteins that form the trimeric viral envelope spike (Env) (20, 34, 45). As improved vaccine immunogens enter the stage of detailed preclinical analysis, the *in vitro* assays used for evaluating vaccine sera will need to detect incremental advances in the magnitude, breadth, and durability of NAb responses (37). Such data can then be used to distinguish and prioritize among antibody-based vaccine immunogens. Furthermore, highly reproducible and quantitative data on vaccine-elicited NAbs can enhance our understanding of the relationship between Env immunogen design and the resulting antibody response generated.

Current recommendations for evaluating candidate vaccine sera for NAb activity include the use of standard reference panels of molecularly cloned HIV-1 Env pseudoviruses and a tiered algorithm of testing (27). Reference virus panels should represent genetically and geographically diverse subsets of viruses with neutralization phenotypes that are generally representative of primary isolate strains that a vaccine would need to protect against. As such, standard reference panels for HIV-1 subtypes B and C have been described (22, 23), and efforts continue toward the creation of virus reference panels representing additional genetic subtypes. For tiered evaluation of NAb activity, vaccine sera are first tested against homologous Env pseudoviruses and/or a small number of isolates that are known to be highly sensitive to antibody-mediated neutralization (commonly referred to as tier 1 viruses). A more rigorous assessment of the potency and breadth of vaccine-induced NAbs entails testing against more resistant reference panel viruses (commonly referred to as tier 2 viruses) that are either matched or mismatched in genetic subtype to the vaccine immunogen (second and third tiers of testing, respectively). This tiered approach for testing candidate HIV-1 vaccine sera is

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$^{\nabla}$ Published ahead of print on 25 November 2009.

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advantageous in that it provides increasingly stringent levels for assessing the potency and breadth of NAbs, uses standardized panels of reference viruses for consistency and reproducibility, and allows for the generation of comparative data sets for evaluating different candidate vaccine regimens.

While the tiered algorithm for evaluating vaccine sera has gained acceptance in the field, a major limitation has been the lack of objective data to characterize HIV-1 Env pseudoviruses according to their overall sensitivity or resistance to antibody-mediated neutralization. The category of sensitive, tier 1 viruses arose in part from the observation that HIV-1 isolates passaged through T-cell lines often become highly sensitive to antibody-mediated neutralization (33). Compared to these laboratory-adapted viruses, most primary isolate strains are moderately resistant to NAbs. Yet, even among recently isolated circulating viral Envs, there is a wide spectrum of neutralization sensitivity. Some HIV-1 isolates have a neutralization phenotype closer to that of tier 1 viruses, while others appear to be quite neutralization resistant (6, 19, 22, 23). Overall, there are few data from which to understand or categorize the viral neutralization phenotypes of HIV-1 strains. As a result, we have a limited ability to assess the potential potency of vaccine-elicited NAbs or to estimate the percentage of circulating HIV-1 isolates that would be neutralized. Further categorization of isolates into distinct subgroups based on sensitivity to NAbs may reveal patterns of neutralization that could provide a greater understanding of the NAb response generated by current and future vaccine immunogens. In addition, the structure-based design of novel immunogens may be facilitated by an ability to monitor the types of viruses neutralized and to specifically map the viral epitopes targeted by vaccine-elicited NAbs.

In this study, we assembled a diverse panel of 109 HIV-1 Env pseudoviruses, including multiple representatives from clades A, B, and C and circulating recombinant forms (CRFs) CRF07_BC and CRF02_AG-related. These were tested for their sensitivities using HIV-1-positive (HIV-1$^+$) plasma samples representative of clades A, B, and C and CRF01_AE and CRF02_AG. Clinical, demographic, and viral genetic sequence data were collected for each virus. The neutralization phenotype of each virus was assessed with a panel of seven clade-specific HIV-1$^+$ plasma pools. Viruses were rank ordered according to average neutralization sensitivity, and *k*-means clustering was utilized to identify four subgroups of viruses with neutralization phenotypes ranging from highly sensitive to resistant. Together, these results will improve the ability to rigorously evaluate antibody-based HIV-1 vaccines and will facilitate the interpretation of assay results to identify immunogens with improved capacity to elicit broadly cross-reactive NAbs.

MATERIALS AND METHODS

Cell lines. The TZM.bl cell line was obtained through the NIH AIDS Research and Reference Reagent Program (ARRRP), as contributed by J. Kappes and X. Wu (12, 36). 293T/17 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Both of these adherent cell lines were maintained in Dulbecco's modified Eagle's medium (D-MEM) growth medium (Gibco/Invitrogen) containing 10% heat-inactivated fetal bovine serum (FBS), 25 mM HEPES, and 50 μg/ml gentamicin. Cells were harvested using trypsin/EDTA solution (Gibco/Invitrogen). All cell lines were maintained at 37°C in humidified air containing 5% CO_2.

Plasma samples, HIV immunoglobulin (HIVIG), and monoclonal antibodies (MAbs). Plasma samples from HIV-1-infected donors not on antiretroviral therapy were obtained from various sources. Four subtype-B plasma samples (1648, 1652, 1686, and 1702) which comprised the clade B-Zepto pool were obtained from Zeptometrix Corporation (Buffalo, NY). Five subtype-C plasma samples (BB12, BB34, BB55, BB87, and BB107) which comprised the clade C-SA pool (South Africa) were purchased from the South African National Blood Services (Johannesburg) and provided by Lynn Morris. The above-mentioned plasma samples were selected based on previous characterization of HIV-1 neutralizing antibody activity (4) and were assayed against all viruses individually or as a pool containing an equal mixture of all constituents. The clade A pool (East Africa), clade B pool (United States), clade C-TZ pool (Tanzania), CRF01_AE pool (Thailand), and CRF02_AG pool (Cameroon) were generously provided by Victoria Polonis (Walter Reed Army Institute of Research). Each pool consisted of an equal mixture of 6 individual plasma samples, with the exception of the clade B pool, which contained a mixture of approximately 600 plasma samples. All constituents of the clade A, clade C-TZ, and CRF01_AE pools were confirmed as pure subtype infections by full HIV-1 genome sequencing, whereas constituents of the clade B and CRF02_AG pool were selected based on likely subtype of infection. Donor clinical information was not available for the plasma samples used in these studies, but all were likely obtained during the chronic phase of HIV-1 infection. Two HIV-1-negative control plasma samples were obtained from Zeptometrix. All plasma samples were heat inactivated for 1 h at 56°C prior to use in neutralization assays.

Polyclonal HIVIG was obtained from the NIH ARRRP. The MAbs 17b and 1.5E, directed against the coreceptor and CD4 binding site (CD4bs) on gp120, respectively, were provided by James Robinson (Tulane University). The anti-V3 loop MAb 447-52D was provided by Susan Zolla-Pazner (New York University).

Viral isolates. Molecularly cloned gp160 genes for HIV-1 Env pseudovirus production were obtained as follows: the subtype B Env clone SF162.LS (41) was obtained from Leonidas Stamatatos (Seattle Biomedical Research Institute). Env clones BaL.26 (40) and HxB2.DG (14) (both subtype B) and DJ263.8 (24) (subtype CRF02_AG) were provided by John Mascola (NIH/Vaccine Research Center). Clones BZ167.12 and Bx08.16 (both subtype B) were obtained from infected peripheral blood mononuclear cell (PBMC) DNA as described previously (22) by using parental viruses obtained from the NIH ARRRP. Clones representing the standard panel of subtype B HIV-1 reference strains (6535.3, QH0692.42, SC422661.8, PVO.4, TRO.11, AC10.0.29, RHPA4259.7, THRO4156.18, REJO4541.67, TRJO4551.58, WITO4160.33, and CAAN5342.A2) and the standard panel of subtype C HIV-1 reference strains (Du156.12, Du172.17, Du422.1, CAP45.2.00.G3, CAP210.2.00.E8, ZM197M.PB7, ZM214M.PL15, ZM233M.PB6, ZM249M.PL1, ZM53M.PB12, ZM109F.PB4, and ZM135M.PL10a) have been previously described (22, 23). These clones, as well as SS1196.1 (subtype B) and MW965.26, Du123.6, and Du151.2 (all subtype C) were obtained from the NIH ARRRP. The panel of recently transmitted subtype C clones from India (HIV-00836-2.5, HIV-001428-2.42, HIV-0013095-2.11, HIV-16055-2.3, HIV-16845-2.22, HIV-16936-2.21, HIV-25710-2.43, HIV-25711-2.4, HIV-25925-2.22, and HIV-26191-2.48) have recently been described (19) and are available through the NIH ARRRP. The panel of transmitted subtype B clones (WEAU-d15.410.787, 1006-11.C3.1601, 1054-07.TC4.1499, 1056-10.TA11.1826, 1012-11.TC21.3257, 6240.08.TA5.4622, 6244.13.B5.4576, 62357.14.D3.4589, 9021.12.B2.4571, 700010040.C9.4520, PRB926-04.A9.4237, and SC05.8C11.2344) were provided by George Shaw and Beatrice Hahn (18). The six Kenyan acute/early subtype A Env clones (Q23ENV17, Q461ENVe2, Q769ENVd22, Q259ENVd2.17, Q842ENVd12, and Q168ENVa2) were obtained from the NIH ARRRP as contributed by Julie Overbaugh (26, 38). Additional acute/early clade A clones from Tanzania (3415.v1.c1, 3365.v2.c20, 0260.v5.c1, 3718.v3.c11, 0439.v5.c1, and 0330.v4.c3) were provided by Francine McCutchan (Henry M. Jackson Foundation). Env clones from sexually transmitted subtype B chronic isolates (H022.7, H029.12, H030.7, H031.7, H035.18, H061.14, H079.2, H086.8, H077.31, H078.14, and H080.23) from Lima, Peru, were obtained from infected PBMC DNA as described previously (22), by using specimens contributed by Rosario Zuniga and Christian Brander. The panel of chronic CRF07_BC gp160 clones from China (CH181.12, CH064.20, CH117.4, CH119.10, CH110.2, CH114.8, CH120.6, CH115.12, CH070.1, and CH111.8, CH038.12) are from intravenous drug use transmissions and were provided by Yiming Shao and Kunxue Hong (Chinese Center for Disease Control and Prevention, Beijing, China). Env clones from Cameroon (257-31, 252-7, 33-7, 263-8, 250-4, 251-18, 278-50, 255-34, 266-60, 253-11, 280-5, 271-11, 211-9, 235-47, 242-14, and 269-12) and Cote d'Ivoire (928-28) were obtained from Dennis Ellenberger (CDC, Atlanta, GA). More detailed information on all HIV-1 gp160 Env clones used in this study can be found in Table 1, as well as the Los Alamos National Laboratories website (www.hiv.lanl.gov/content/nab-reference-strains).

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TABLE 1. HIV-1 Env pseudovirus panel

HIV-1 isolate[a]	Tier	Subtype[b]	Country of origin	Stage of infection[c]	Mode of transmission[d]	Source[e]	GenBank accession no.[f]	ID_{50} titer (μg/ml)[g]			
								HIVIG	447-52D	17b	1.5E
MW965.26	1A	C	Malawi	Fiebig VI	M-F	ccPBMC	U08455	**<0.02**	**0.05**	**0.15**	>50
SF162.LS	1A	B	USA	Fiebig VI	M-M	CSF	EU123924	**6**	**<0.02**	**0.8**	**2.3**
MN/H9	1A	B	USA	Fiebig VI	Mother to child	TCLA	NA	**7**	**<0.02**	**0.2**	**25.4**
DJ263.8	1B	02_AG	Djibouti	Fiebig VI	Sexual	ccPBMC	AF063223	**89**	**20.5**	**48.3**	>50
00836-2.5	1B	C	India	Fiebig VI	M-F	ccPBMC	EF117265	**455**	>50	>50	>50
Bx08.16	1B	B	France	Fiebig VI	Unknown	ccPBMC	AY713411	**81**	**0.1**	**0.9**	**10.1**
BaL.26	1B	B	USA	Fiebig VI	Mother to child	Lung	DQ318211	**69**	**0.04**	**11.4**	**15.6**
HXB2.DG	1B	B	France	AIDS	M-M	TCLA	K03455	**28**	**<0.02**	**1.0**	**1.0**
271-11	1B	02_AG	Cameroon	Acute/early	Unknown	ucPBMC	EU513197	**344**	>50	**18.6**	>50
242-14	1B	02A1	Cameroon	Fiebig VI	Unknown	ccPBMC	EU513188	**583**	>50	>50	>50
25710-2.43	1B	C	India	Fiebig V	F-M	ccPBMC	EF117271	**311**	>50	>50	>50
H077.31	1B	B	Peru	Fiebig VI	Sexual	ccPBMC	EF210734	**783**	>50	>50	>50
6535.3	1B	B (ref.)	USA	Fiebig V	M-M	ccPBMC	AY835438	**264**	**0.1**	>50	**10.4**
SS1196.1	1B	B	USA	Fiebig V–VI	M-M	ccPBMC	AY835442	**191**	**0.4**	**6.3**	**41.9**
ZM197 M.PB7	1B	C (ref.)	Zambia	Fiebig ≤VI	F-M	ucPBMC	DQ388515	**527**	>50	>50	>50
H029.12	1B	B	Peru	Fiebig VI	Sexual	ccPBMC	EF210726	**267**	>50	>50	>50
1012-11.TC21.3257	1B	B (transm.)	USA	Fiebig III	Unknown	Plasma	EU289184	**559**	>50	>50	>50
H086.8	1B	B	Peru	Fiebig VI	Sexual	ccPBMC	EF210732	**1,141**	>50	>50	>50
Q23ENV17	1B	A1	Kenya	Fiebig VI	FSW	ccPBMC	AF004885	**428**	>50	>50	>50
BZ167.12	1B	B	Brazil	Fiebig VI	Unknown	ccPBMC	AY173956	**184**	**0.2**	>50	>50
25711-2.4	1B	C	India	Fiebig III	F-M	ccPBMC	EF117272	**1,307**	>50	>50	>50
ZM109F.PB4	1B	C (ref.)	Zambia	Fiebig ≤VI	M-F	ucPBMC	AY424138	**1,109**	>50	>50	>50
25925-2.22	1B	C	India	Fiebig III	F-M	ccPBMC	EF117273	**909**	>50	>50	>50
1056-10.TA11.1826	1B	B (transm.)	USA	Fiebig II	Unknown	Plasma	EU289186	**522**	>50	>50	>50
16845-2.22	2	C	India	Fiebig V	M-F	ccPBMC	EF117269	**678**	>50	>50	>50
Du156.12	2	C	South Africa	Fiebig ≤IV	FSW	ccPBMC	DQ411852	**770**	>50	>50	>50
TRO.11	2	B (ref.)	Italy	Fiebig III	M-M	ccPBMC	AY835445	**705**	>50	>50	>50
26191-2.48	2	C	India	Fiebig III	F-M	ccPBMC	EF117274	**2,439**	>50	>50	>50
SC422661.8	2	B (ref.)	Trinidad and Tobago	Fiebig IV	F-M	Plasma	AY835441	**662**	>50	>50	>50
WITO4160.33	2	B (ref.)	USA	Fiebig II	F-M	Plasma	AY835451	**764**	>50	>50	>50
0013095-2.11	2	C	India	Fiebig IV	M-F	ccPBMC	EF117267	**400**	>50	>50	>50
REJO4541.67	2	B (ref.)	USA	Fiebig II	F-M	Plasma	AY835449	**324**	>50	>50	>50
ZM135 M.PL10a	2	C	Zambia	Fiebig ≤VI	F-M	Plasma	AY424079	**616**	>50	>50	>50
700010040.C9.4520	2	B (transm.)	USA	Fiebig V	M-M	Plasma	EU289193	**812**	>50	>50	>50
Du123.6	2	C	South Africa	Fiebig VI	FSW	ccPBMC	DQ411850	**991**	>50	>50	>50
ZM249 M.PL1	2	C (ref.)	Zambia	Fiebig II	F-M	Plasma	DQ388514	**881**	>50	>50	>50
266-60	2	02_AG	Cameroon	Fiebig VI	Unknown	ucPBMC	EU513193	**1,179**	>50	>50	>50
16936-2.21	2	C	India	Fiebig III	F-M	ccPBMC	EF117270	**829**	>50	>50	>50
Q842ENVd12	2	A1	Kenya	Acute/early	FSW	ucPBMC	AF407160	**425**	>50	>50	>50
SC05.8C11.2344	2	B (transm.)	Trinidad and Tobago	Fiebig II	F-M	Plasma	EU289200	**577**	>50	>50	>50
PRB926-04.A9.4237	2	B (transm.)	USA	Fiebig II	Unknown	Plasma	EU289197	**318**	>50	>50	>50
Q259ENVd2.17	2	A1	Kenya	Acute/early	FSW	ucPBMC	AF407152	**695**	>50	>50	>50
255-34	2	02_AG	Cameroon	Fiebig VI	Unknown	ccPBMC	EU513184	**1,137**	>50	>50	>50
H022.7	2	B	Peru	Fiebig VI	Sexual	ccPBMC	EF210725	>2,500	>50	>50	>50
Q769ENVd22	2	A1	Kenya	Acute/early	FSW	ucPBMC	AF407158	**658**	>50	>50	>50
1006-11.C3.1601	2	B (transm.)	USA	Fiebig III	Unknown	Plasma	EU289183	**622**	>50	>50	>50
280-5	2	02A1U	Cameroon	Fiebig VI	Unknown	ucPBMC	EU513183	**639**	>50	>50	>50
CAP210.2.00.E8	2	C (ref.)	South Africa	Fiebig IV	FSW	Plasma	DQ435683	**913**	>50	>50	>50
WEAU-d15.410.787	2	B (transm.)	USA	Fiebig II	M-M	Plasma	EU289202	**616**	>50	>50	>50
263-8	2	A1U	Cameroon	Fiebig VI	Unknown	ccPBMC	EU513182	**611**	>50	>50	>50
0330.v4.c3	2	A	Tanzania	Fiebig V/VI	M-F	Plasma	NA	**845**	>50	>50	>50
AC10.0.29	2	B (ref.)	USA	Fiebig III	M-M	ccPBMC	AY835446	**659**	>50	>50	>50
3415.v1.c1	2	A	Tanzania	Fiebig V/VI	F-M	Plasma	NA	**688**	>50	>50	>50
6240.08.TA5.4622	2	B (transm.)	USA	Fiebig II	Unknown	Plasma	EU289190	**1224**	>50	>50	>50
252-7	2	U	Cameroon	Fiebig VI	Unknown	ucPBMC	EU513190	>2,500	>50	>50	>50
CH110.2	2	07_BC	China	Fiebig VI	IDU	ccPBMC	EF117257	**379**	>50	>50	>50
6244.13.B5.4567	2	B (transm.)	USA	Fiebig II	Unknown	Plasma	EU289191	**664**	>50	>50	>50
Q168ENVa2	2	A1	Kenya	Acute/early	FSW	ucPBMC	AF407148	**926**	>50	>50	>50
235-47	2	02_AG	Cameroon	Fiebig VI	Unknown	ucPBMC	EU513195	**1,112**	>50	>50	>50
62357.14.D3.4589	2	B (transm.)	USA	Fiebig II	Unknown	Plasma	EU289189	**730**	>50	>50	>50
CAAN5342.A2	2	B (ref.)	USA	Fiebig ≤VI	M-M	Plasma	AY835452	**1,134**	>50	>50	>50
H080.23	2	B	Peru	Fiebig VI	Sexual	ccPBMC	EF210735	**1,506**	>50	>50	>50
Du172.17	2	C	South Africa	Fiebig VI	FSW	ccPBMC	DQ411853	**1,216**	>50	>50	>50
Du151.2	2	C	South Africa	Fiebig V	FSW	ccPBMC	DQ411851	**1,091**	>50	>50	>50
ZM233 M.PB6	2	C (ref.)	Zambia	Fiebig ≤VI	F-M	ucPBMC	DQ388517	**734**	>50	>50	>50
CAP45.2.00.G3	2	C (ref.)	South Africa	Fiebig IV	FSW	Plasma	DQ435682	**1,813**	>50	>50	>50
ZM214 M.PL15	2	C (ref.)	Zambia	Fiebig ≤VI	F-M	Plasma	DQ388516	>2,500	>50	>50	>50
0439.v5.c1	2	A	Tanzania	Fiebig V/VI	M-F	Plasma	NA	**1,283**	>50	>50	>50
269-12	2	02U	Cameroon	Fiebig VI	Unknown	ccPBMC	EU513194	**1,706**	>50	>50	>50
9021.14.B2.4571	2	B (transm.)	USA	Fiebig II	Unknown	Plasma	EU289196	**585**	>50	>50	>50
H030.7	2	B	Peru	Fiebig VI	Sexual	ccPBMC	EF210727	**2,165**	>50	>50	>50
3718.v3.c11	2	A	Tanzania	Fiebig I/II	M-F	Plasma	NA	>2,500	>50	>50	>50
0260.v5.c1	2	A	Tanzania	Fiebig V/VI	F-M	Plasma	NA	**623**	>50	>50	>50
247-23	2	DU	Cameroon	Not recorded	Unknown	ucPBMC	EU683891	**815**	>50	>50	>50
QH0692.42	2	B (ref.)	Trinidad and Tobago	Fiebig V	F-M	ccPBMC	AY835439	**1,812**	**39.3**	>50	>50
THRO4156.18	2	B (ref.)	USA	Fiebig II	M-M	Plasma	AY835448	**841**	>50	**32.1**	**40.3**

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TABLE 1—*Continued*

HIV-1 isolate[a]	Tier	Subtype[b]	Country of origin	Stage of infection[c]	Mode of transmission[d]	Source[e]	GenBank accession no.[f]	ID_{50} titer (μg/ml)[g]			
								HIVIG	447-52D	17b	1.5E
3365.v2.c20	2	A	Tanzania	Fiebig V/VI	M-F	Plasma	NA	**1361**	>50	>50	>50
ZM53 M.PB12	2	C (ref.)	Zambia	Fiebig ≤VI	F-M	ucPBMC	AY423984	**1,903**	>50	>50	>50
1054-07.TC4.1499	2	B (transm.)	USA	Fiebig II	Unknown	Plasma	EU289185	**1157**	>50	>50	>50
16055-2.3	2	C	India	Fiebig II	F-M	ccPBMC	EF117268	>2,500	>50	>50	>50
RHPA4259.7	2	B (ref.)	USA	Fiebig ≤V	M-F	Plasma	AY835447	**544**	**48.9**	>50	>50
CH181.12	2	07_BC	China	Fiebig VI	IDU	ccPBMC	EF117259	**1,534**	>50	>50	>50
250-4	2	02_AG	Cameroon	Fiebig VI	Unknown	ccPBMC	EU513189	**1,072**	>50	>50	>50
001428-2.42	2	C	India	Fiebig IV	M-F	ccPBMC	EF117266	**660**	>50	>50	>50
Du422.1	2	C (ref.)	South Africa	Fiebig V	FSW	ccPBMC	DQ411854	**949**	>50	>50	>50
CH064.20	2	07_BC	China	Fiebig VI	IDU	ccPBMC	EF117254	**1,202**	>50	>50	>50
H031.7	2	B	Peru	Fiebig VI	Sexual	ccPBMC	EF210728	**775**	**0.6**	>50	>50
CH111.8	2	07_BC	China	Fiebig VI	IDU	ccPBMC	EF117258	**2,429**	>50	>50	>50
CH119.10	2	07_BC	China	Fiebig VI	IDU	ccPBMC	EF117261	**2,236**	>50	>50	>50
Q461ENVe2	2	A1	Kenya	Acute/early	FSW	ucPBMC	AF407156	>2,500	>50	>50	>50
211-9	2	02_AG	Cameroon	Fiebig VI	Unknown	ccPBMC	EU513187	**2,241**	>50	>50	>50
CH117.4	2	07_BC	China	Fiebig VI	IDU	ccPBMC	EF117262	**836**	>50	>50	>50
H035.18	3	B	Peru	Fiebig VI	Sexual	ccPBMC	EF210729	>2,500	>50	>50	>50
928-28	2	02_AG	Cote d'Ivoire	Acute/early	FSW	ccPBMC	EU513199	>2,500	>50	>50	>50
CH070.1	3	07_BC	China	Fiebig VI	IDU	ccPBMC	EF117255	>2,500	>50	>50	>50
CH038.12	3	07_BC	China	Fiebig VI	IDU	ccPBMC	EF042692	**1,219**	>50	>50	>50
CH114.8	3	07_BC	China	Fiebig VI	IDU	ccPBMC	EF117264	**1,595**	>50	>50	>50
H061.14	3	B	Peru	Fiebig VI	Sexual	ccPBMC	EF210730	**562**	**23.4**	>50	>50
253-11	3	CRF02_AG	Cameroon	Fiebig VI	Unknown	ucPBMC	EU513191	>2,500	>50	>50	>50
257-31	3	02_AG	Cameroon	Acute/early	Unknown	ccPBMC	EU513185	>2,500	>50	>50	>50
PVO.4	3	B (ref.)	Italy	Fiebig III	M-M	ccPBMC	AY835444	**1,277**	>50	>50	>50
TRJO4551.58	3	B (ref.)	USA	Fiebig II	M-M	Plasma	AY835450	**1,221**	>50	>50	>50
H078.14	3	B	Peru	Fiebig VI	Sexual	ccPBMC	EF210733	**1,702**	>50	>50	>50
278-50	3	02A1U	Cameroon	Fiebig VI	Unknown	ccPBMC	EU513198	>2,500	>50	>50	>50
H079.2	3	B	Peru	Fiebig VI	Sexual	ccPBMC	EF210731	**1,445**	>50	>50	>50
CH115.12	3	07_BC	China	Fiebig VI	IDU	ccPBMC	EF117263	>2,500	>50	>50	>50
33-7	3	02A1U	Cameroon	Fiebig VI	Unknown	ccPBMC	EU513186	**1,900**	>50	>50	>50
CH120.6	3	07_BC	China	Fiebig VI	IDU	ccPBMC	EF117260	>2,500	>50	>50	>50
251-18	3	02_AG	Cameroon	Fiebig VI	Unknown	ccPBMC	EU513196	>2,500	>50	>50	>50

[a] HIV-1 isolates are ordered by neutralization sensitivity as determined by average $\log_{10}$ ID_{50} titers.
[b] Subtypes from LANL database. ref., standard reference panel viruses; transm., transmitted/founder viruses.
[c] Fiebig stage as previously defined (13).
[d] M-F, male-to-female; F-M, female-to-male; M-M, male-to-male; FSW, female sex worker; IDU, intravenous drug use.
[e] ccPBMC, cocultured PBMC; ucPBMC, uncultured PBMC; CSF, cerebrospinal fluid.
[f] NA, not available.
[g] Values are the concentration (μg/ml) at which RLU were reduced by 50% compared to the level in virus control wells. Data in bold indicate positive neutralization.

HIV-1 Env pseudovirus production and titration. Stocks of single-round-infection HIV-1 Env pseudovirus were produced by cotransfecting 293T/17 cells (1.7×10^7 cells per T75 flask) with 2 μg of an HIV-1 *rev/env* expression plasmid and 12 μg of an *env*-deficient HIV-1 backbone plasmid (pSG3ΔEnv) using Lipofectamine transfection reagent (Invitrogen). Pseudovirus-containing supernatant was harvested 24 h following transfection and clarified by centrifugation and 0.45-μm filtration. Single-use aliquots (1.0 ml) were stored at −80°C. The 50% tissue culture infectious dose ($TCID_{50}$) for each pseudovirus preparation was determined by infection of TZM.bl cells as previously described (22). A T-cell-line-adapted (TCLA) strain of HIV-1 MN was obtained from the NIH ARRRP as contributed by R. Gallo (15, 39), and cell-free stocks were generated using H9 cells as previously described (32).

Neutralization assay. Virus neutralization was measured using a luciferase-based assay in TZM.bl cells as previously described (31). This assay measures the reduction in luciferase reporter gene expression in TZM.bl cells following a single round of virus infection. Briefly, 3-fold serial dilutions of serum samples were performed in duplicate (96-well flat bottom plate) in 10% D-MEM growth medium (100 μl/well). An amount of 200 $TCID_{50}$ of virus was added to each well in a volume of 50 μl, and the plates were incubated for 1 h at 37°C. TZM.bl cells were then added (1×10^4/well in a 100-μl volume) in 10% D-MEM growth medium containing DEAE-dextran (Sigma, St. Louis, MO) at a final concentration of 11 μg/ml. Assay controls included replicate wells of TZM.bl cells alone (cell control) and TZM.bl cells with virus (virus control). Following a 48-h incubation at 37°C, 150 μl of assay medium was removed from each well and 100 μl of Bright-Glo luciferase reagent (Promega, Madison, WI) was added. The cells were allowed to lyse for 2 min, and then 150 μl of the cell lysate was transferred to a 96-well black solid plate, and luminescence was measured using a Victor 3 luminometer (Perkin Elmer). The 50% inhibitory dose (ID_{50}) titer was calculated as the serum dilution that caused a 50% reduction in relative luminescence units (RLU) compared to the level in the virus control wells after subtraction of cell control RLU. All data were analyzed with 5-parameter curve fitting using neutralizing antibody analysis software provided by the CAVD Vaccine Immunology Statistical Center.

Statistical analysis. Viruses were compared with respect to overall neutralization sensitivity by rank ordering based on average $\log_{10}$ ID_{50} titers across the 7 plasma pools. Viruses were grouped using *k*-means clustering, with the number of clusters determined using the methods of Tibshirani et al. (42) and Calinski and Harabasz (10). A heatmap that illustrates the data underlying the clustering patterns was generated using a modified version of the heatmap tool on the Los Alamos database (http://www.hiv.lanl.gov/content/sequence/HEATMAP/heatmap.html). The number of clusters that appear in the heatmap was determined by successively increasing the number of clusters used in a *k*-means analysis and stopping at the maximum number of clusters that achieved a threshold level of stability. The robustness of membership within a given cluster was evaluated by bootstrap resampling of the pooled sera and, also, by including a model of assay-to-assay variation based on repeat experiments for a subset of samples. Cluster membership was considered stable if it was seen in at least 80% of the bootstrap and model samples; only four clusters were found to be stable at this threshold. The impact of virus characteristics on neutralization sensitivity was evaluated using a linear generalized estimating equation (GEE) model for $\log_{10}$ ID_{50} titers (25), with the inclusion of a binary covariate to indicate whether the virus and plasma pool were of the same clade. The relationships between the NAb activities of plasma pools and those of their constituents were evaluated by calculating the average ID_{50} titer value across all the constituents in each pool, log transforming this average, and comparing it with the $\log_{10}$ ID_{50} value for the plasma pool. Phylogenetic analysis used the maximum-likelihood method as previously described (3).

RESULTS

Characteristics of the assembled viral isolates and HIV-1$^+$ plasma samples. A panel of 109 Env pseudoviruses was assem-

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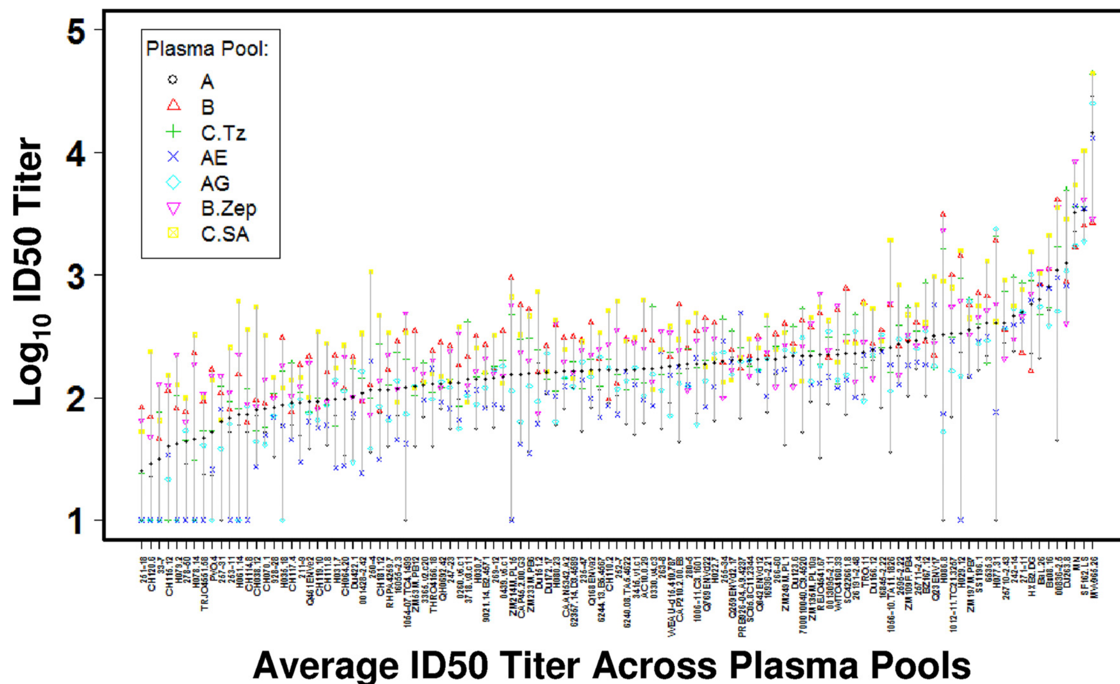

FIG. 1. Ordering of HIV-1 isolates based on average neutralization sensitivities. HIV-1 Env pseudoviruses (n = 109) were assessed for neutralization sensitivities using seven pools of subtype-specific HIV-1$^+$ plasma from chronically infected individuals. Viruses are rank ordered from lowest (least sensitive) to highest (most sensitive) on the x axis according to the average $\log_{10}$ ID_{50} titer from all seven plasma pools. The ID_{50} titer for each plasma pool is indicated on the y axis by the representative symbol, with the pools having the highest and lowest ID_{50} titers for each virus connected by a vertical line. The black circles indicate the average ID_{50} titer across all seven plasma pools. ID_{50} titers below the limit of detection (1:20) were assigned a value of 10 for this figure.

bled for characterizing the spectrum of HIV-1 sensitivities to antibody-mediated neutralization. Viruses were primarily chosen based on genetic diversity (predominantly subtypes A, B, C, CRF07_BC, and CRF02_AG-related), geographic diversity (United States, Europe, South Africa, Tanzania, Ethiopia, Zambia, Cameroon, Peru, India, and China), and stage of infection (transmitted/founder, early/acute, and chronic). The vast majority of isolates were from sexually acquired infections, and a smaller subset (approximately 10%, all subtype CRF07_BC) was from intravenous drug use transmissions. Viruses comprising recommended panels of clade B and clade C reference strains (22, 23), as well as isolates known historically to be highly sensitive to antibody-mediated neutralization, were also included. More detailed information for each of these viruses is presented in Table 1.

To assess and compare the relative neutralization sensitivities of these viruses, we utilized diverse panels of HIV-1$^+$ plasma pools for NAb assays. The clade A pool (East Africa), clade B pool (United States), clade C-TZ pool (Tanzania), CRF01_AE pool (Thailand), and CRF02_AG pool (Cameroon) were each constructed using equal amounts of plasma from at least six chronically infected donors not on antiretroviral therapy. No preselection criteria (i.e., prior NAb data) were used for choosing constituent plasma samples to include in these plasma pools. We also constructed an additional clade B-Zepto pool (Zeptometrix) and clade C-SA pool (South Africa) using constituent plasma samples that were selected based on extensive prior characterization and known broad NAb activity (4). The few existing MAbs that neutralize primary HIV-1 isolates were not used to categorize viruses in this study because these were derived solely from clade B-infected subjects and are directed to a limited set of epitopes on HIV-1 gp120 and gp41.

Ordering of HIV-1 isolates by average $\log_{10}$ ID_{50} titer. NAb assays were performed to determine the sensitivity of each of the 109 HIV-1 isolates to neutralization by each of the seven HIV-1$^+$ plasma pools. Assays were conducted using a validated NAb assay protocol under full good clinical laboratory practice (GCLP) compliance. The distribution of viruses according to average $\log_{10}$ ID_{50} titer is shown in Fig. 1. While many of the viruses tested exhibited a similar range of average sensitivities to neutralization by the various plasma pools, subsets of viruses at either end of the spectrum clearly demonstrated a more sensitive or resistant phenotype. The three most sensitive viruses (MW965.26, SF162.LS, and MN) were neutralized by all plasma pools with reciprocal $\log_{10}$ ID_{50} titers ranging from 3.0 to 4.5, whereas viruses on the opposite end of the rank order were relatively insensitive to neutralization by several of the seven HIV-1$^+$ plasma pools (ID_{50} titers of <20). Most viruses also demonstrated a variable spectrum of sensitivities to the plasma pools, as shown by the vertical lines in Fig. 1. In general, the clade B, clade C-SA, and clade B-Zepto pools demonstrated higher levels of neutralization potency than the other plasma pools. No significant NAb activity was observed when plasma pools were tested against a negative-control murine leukemia virus (MuLV) or when HIV-1 Env pseudoviruses were assayed using normal naïve plasma (data not shown).

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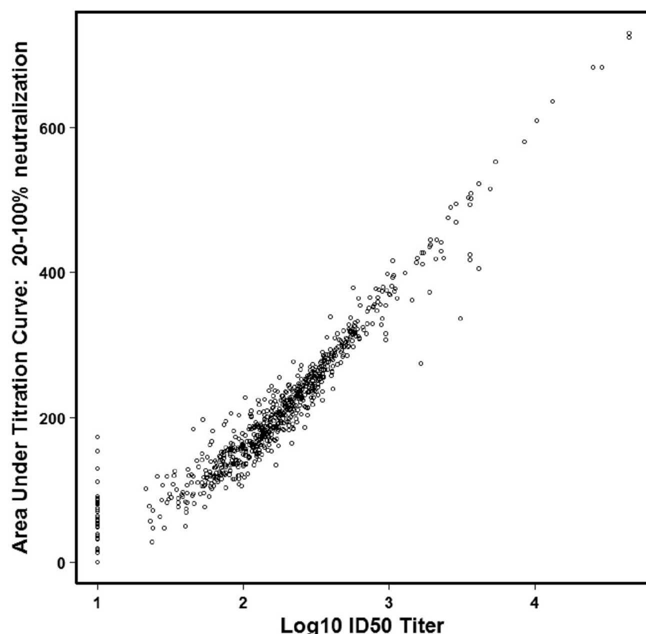

FIG. 2. Correlation between ID_{50} titer and pAUC. Neutralization assays performed for all plasma pools and viruses were analyzed to determine the $\log_{10}$ ID_{50} titer (*x* axis) and the pAUC (20 to 100% neutralization, *y* axis). Each symbol represents neutralization data from a single virus-plasma pool pair.

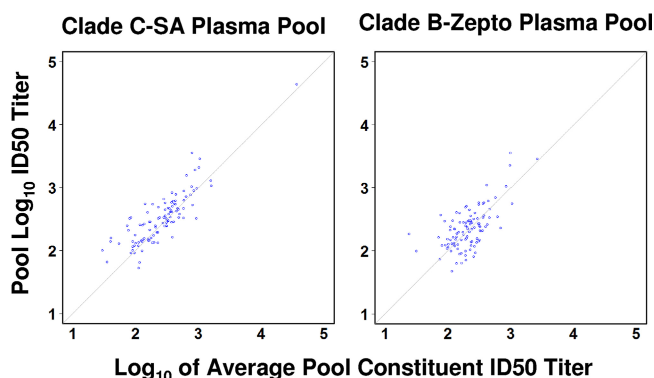

FIG. 3. ID_{50} titers in plasma pools versus average ID_{50} titers of constituents. NAb titers against HIV-1 pseudoviruses were assessed using the clade C-SA pool and the clade B-Zepto pool, as well as each of the individual constituents that make up these respective plasma pools. The $\log_{10}$ ID_{50} titer of each plasma pool (*y* axis) is plotted against the $\log_{10}$ of the average ID_{50} titers of the pool constituents (*x* axis).

In addition to ordering viruses based on NAb ID_{50} titers, we investigated the use of ID_{80} titers and area under the titration curve (AUC) as parameters for assessing neutralization sensitivity. As many HIV-1^+ plasma pools did not achieve at least 80% neutralization against a large proportion of viruses at the lowest dilution tested (1:20), the high number of left-censored values (approximately 33%) made interpretation of ID_{80} rank-ordering results difficult (data not shown). We hypothesized that AUC analysis of the NAb assay data would prove a useful method for ordering viruses, as every virus/plasma pool test provides a measurable data value regardless of whether 50% or 80% neutralization is achieved. To minimize the influence of titration curve area that is within the realm of noise in the NAb assay, we performed all analyses using a partial AUC (pAUC), defined as the area under the titration curve measured between 20 and 100% neutralization. The ID_{50} titer and pAUC for each virus/plasma pool were found to be highly correlated ($r = 0.95$, $P < 2.2 \times 10^{-16}$) (Fig. 2). While pAUC did provide measurable values for several virus/plasma assays that had undetectable ID_{50} titers, overall the two methods gave very similar results. As such, when viruses were ordered and comparisons performed using pAUC, the results were found to be highly concordant with the average $\log_{10}$ ID_{50} titer ordering analysis (data not shown).

Comparison of neutralization activities in plasma pools versus those of individual constituents of the pools. To maximize the diversity of plasma samples used for assessing the neutralization sensitivities of isolates in our HIV-1 Env pseudovirus panel, we utilized subtype-specific HIV-1^+ plasma pools. The majority of pools were created using an equal mixture of plasma from 4 to 6 individuals, which we reasoned would provide the desired sample diversity of the antibody repertoire without extensively diluting out NAbs present in any one of the constituent samples. To further investigate how the neutralization of a plasma pool related to the neutralization of its constituents and whether the pooling of samples was a reasonable means to represent individual plasma samples, we tested each of the constituent plasma samples represented in the clade B-Zepto pool ($n = 4$) and clade C-SA pool ($n = 5$) in NAb assays against the entire panel of HIV-1 Env pseudoviruses. Since the pools were comprised of equal parts of the constituents, we compared the $\log_{10}$ ID_{50} titer of each plasma pool with the log of the average ID_{50} titer of its constituents. As shown in Fig. 3, we found reasonable agreement between the NAb titers of the plasma pools and the expected average NAb titers of the individual constituents ($r = 0.64$ and $r = 0.85$ for the clade B-Zepto and C-SA samples, respectively) and did not detect any pronounced evidence of synergy or hindrance associated with the pooling of samples.

Neutralization sensitivity of standard reference viruses. Our panel of HIV-1 Env pseudoviruses included recommended panels of clade B and clade C reference viruses that are used extensively for standardized assessments of NAbs ($n = 12$ isolates/panel). The rank-ordering analysis allowed us to better define the spectrum of neutralization sensitivities represented by reference panel viruses in the context of a larger number of HIV-1 Env pseudoviruses. Figure 4 outlines the individual isolates in the standard clade B and clade C reference panels. In addition, we assessed the rank-order distribution of 12 clade A isolates and 12 clade B transmitted/founder viruses. These results show that each of the subpanels represents a reasonable distribution of viruses as determined by rank order and that these reference virus panels should continue to be highly useful as standardized reagents for neutralization assays.

Virus characteristics associated with neutralization sensitivity. Associations were sought between the neutralization sensitivity and other properties of the viruses. Various characteristics examined included clade and CRF (A, B, C, D, CRF02_AG, and CRF07_BC), stage of infection of the individual from whom the Env gene was cloned (transmitted,

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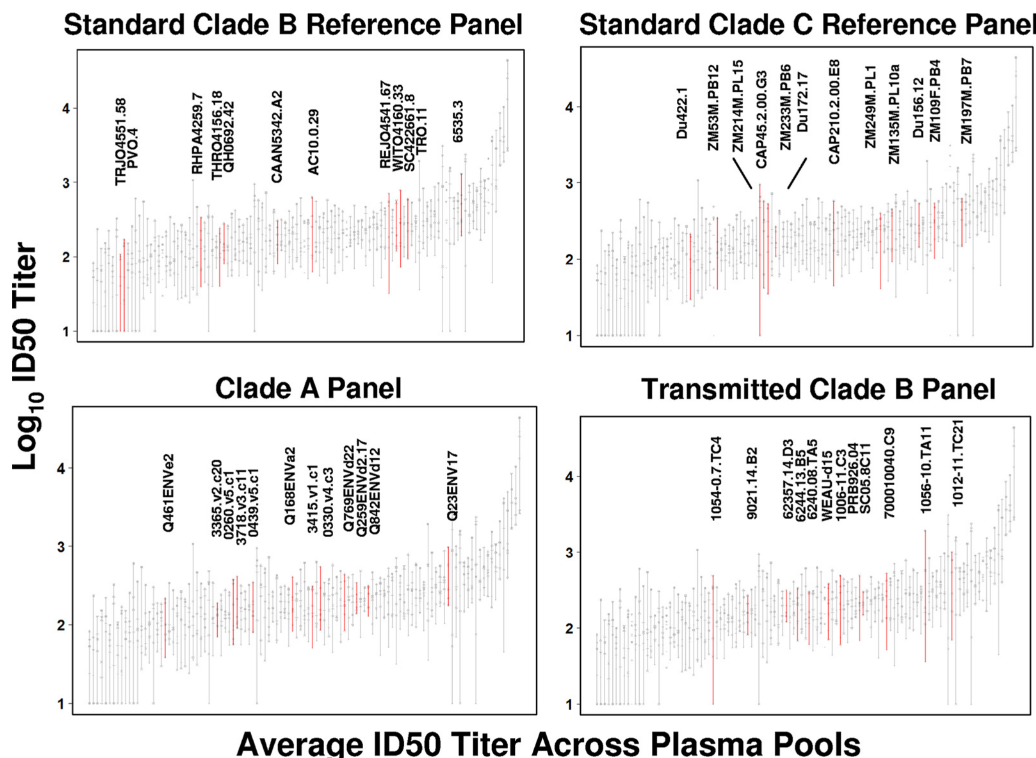

FIG. 4. Rank-order distribution within standard virus reference panels. The rank-order distribution of HIV-1 Env pseudoviruses based on average $\log_{10}$ ID_{50} titers is shown as described for Fig. 1. Individual isolates which constitute the standard clade B reference panel, the standard clade C reference panel, a clade A panel, and a transmitted clade B panel are highlighted in red.

acute/early, or chronic), and the source of virus (plasma, cocultured PBMC, or uncultured PBMC). The effect of clade match between the viral isolate and the plasma pool tested was also examined. A generalized estimating equation model was used to quantify the association between the above-mentioned characteristics and NAb ID_{50} titers (Table 2). While no significant associations were found with the source of virus or stage of infection, we did observe a significant impact of clade (generalized Wald test, $P = 0.0019$). In general, the clade A and clade C isolates of this panel tended to be more sensitive to antibody-mediated neutralization than B isolates, whereas the CRF02_AG and CRF07_BC viruses were less sensitive. In particular, we found that the greatest predictor of neutralization sensitivity was when the clade of the HIV-1^+ plasma pool matched the clade of the virus (generalized Wald test, $P < 0.0001$). The clade A, B, and C HIV-1^+ plasma pools demonstrated greater NAb potency against viruses of the matched clade than against viruses from a nonmatched clade (Fig. 5).

Additional NAb phenotyping of HIV-1 Env pseudoviruses. We further tested all HIV-1 Env pseudoviruses for sensitivity to neutralization by MAbs specific for the V3 loop (MAb 447-52D), the CD4 receptor binding site (MAb 1.5E), or the coreceptor binding site (MAb 17b) of gp120 (11, 17, 47). Isolates susceptible to neutralization by these particular MAbs have often been regarded as globally sensitive strains that may not be representative of most circulating viruses (34). We therefore wanted to assess whether neutralization by 447-52D, 17b, or 1.5E was predictive of neutralization sensitivity as de-

TABLE 2. Association between virus characteristics and neutralization sensitivity

Characteristic[d]	Difference in log ID_{50} (95% CI)[a]	P value[b]
Stage		0.0842
Early	0.00	
Chronic	0.30 (−0.15, 0.75)	
Transmitted	0.24 (0.03, 0.45)	
Source		0.8120
Plasma	0.00	
ccPBMC	0.05 (−0.15, 0.25)	
ucPBMC	−0.01 (−0.18, 0.15)	
Clade		0.0019
B	0.00	
A	0.26 (0.004, 0.52)	
C	0.32 (−0.03, 0.66)	
CRF02_AG[c]	−0.28 (−0.59, 0.02)	
CRF07_BC	−0.42 (−0.70, −0.14)	
D	−0.20 (−0.46, 0.05)	
Virus/plasma clade match	0.25 (0.19, 0.32)	<0.0001

[a] Estimates are derived from a GEE model for $\log_{10}$ ID_{50} titers that includes all virus characteristics as covariates. For each characteristic, the estimated difference in mean $\log_{10}$ ID_{50} titers between each group and the reference group is shown. 95% CI, 95% confidence interval.
[b] P values are based on generalized Wald tests.
[c] CRF02_AG viruses include pure subtype and closely related CRF02 isolates, all from Cameroon (see Table 1 and Fig. 8).
[d] ccPBMC, cocultured PBMC; ucPBMC, uncultured PBMC.

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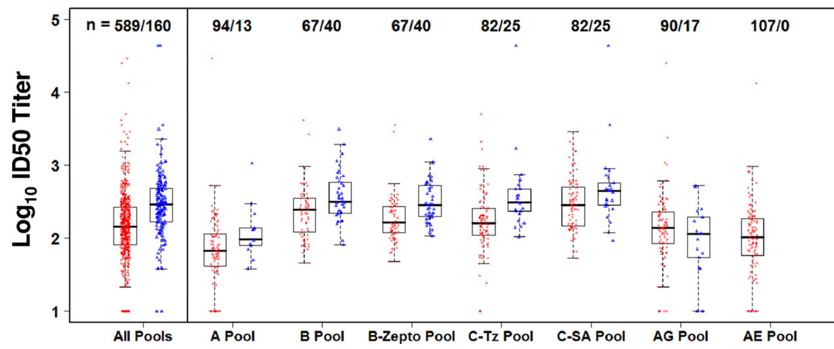

FIG. 5. Higher levels of neutralization activity are observed in the setting of clade match between the virus and plasma sample. For each plasma pool listed, the distribution of $\log_{10}$ ID_{50} titers of viruses either matched (blue triangles) or mismatched (red circles) in clade is shown. Box plots identify the median and interquartile range of the distribution. Box plots on the far left show data combined across all plasma pools. The number of viruses plotted for each group is indicated as number mismatched/number matched. The two strains of TCLA virus (MN and HxB2) were not included in these analyses to avoid potential bias.

termined by our panel of HIV-1^+ plasma samples. In general, the approximate top 10% of sensitive viruses identified in our rank-ordering analysis clearly demonstrated enhanced susceptibility to these particular MAbs, whereas almost all other isolates were resistant (Table 1). However, a few exceptions were noted in which lower rank-ordered viruses (less neutralization sensitive to plasma pools) demonstrated sensitivity to one or more of these MAbs. These data demonstrate that the use of a complex panel of HIV-1^+ plasma samples reveals patterns of virus neutralization that are more complex than those revealed by individual MAbs. Therefore, polyclonal plasma samples may be a more robust method for comparing the global sensitivities of a large panel of HIV-1 viruses and identifying particularly sensitive or resistant isolates.

Grouping of HIV-1 Env pseudoviruses based on patterns in neutralization sensitivity. With the initial rank ordering of HIV-1 Env pseudoviruses as a background, we sought to determine whether these NAb data could be further utilized to group subsets of viruses based on patterns in neutralization sensitivity. *k*-means clustering of NAb ID_{50} titers from each virus/plasma pool combination distinguished four groups of viruses that exhibit similar neutralization profiles (Fig. 6). Two clusters of viruses were identified (designated here as tier 1A and tier 1B) that have a more sensitive neutralization phenotype overall than all other HIV-1 Env pseudoviruses evaluated. The majority of viruses formed a cluster with moderate neutralization sensitivity (tier 2), whereas a small fraction of viruses exhibited a more neutralization-resistant phenotype (tier 3).

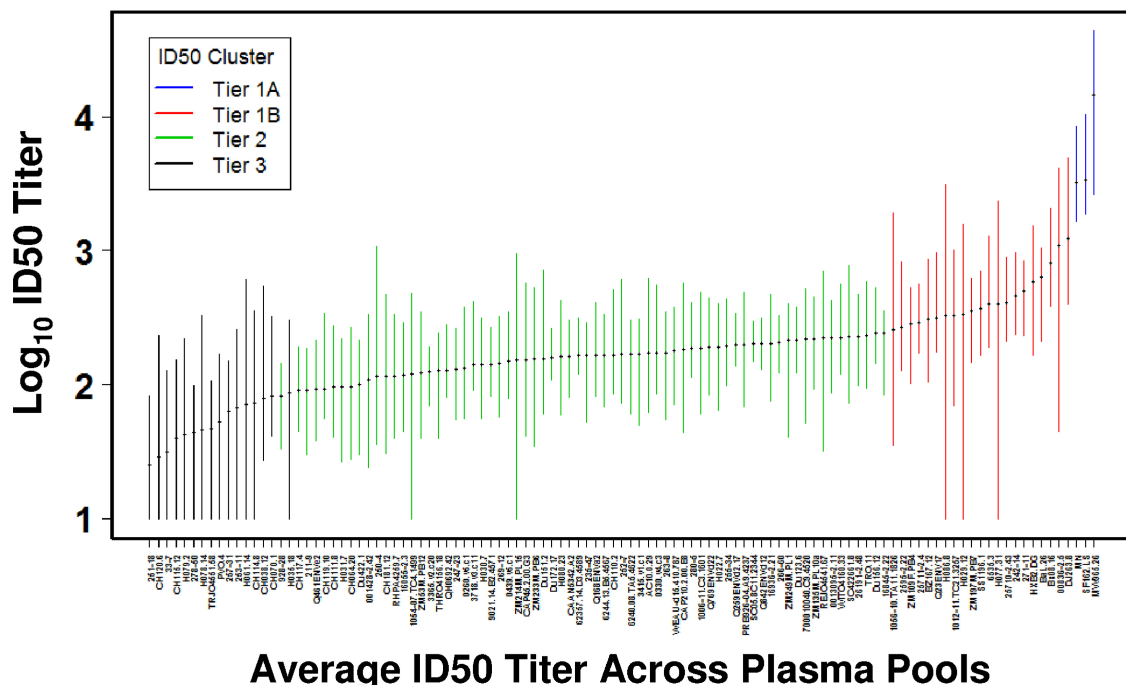

FIG. 6. *k*-means clustering of HIV-1 Env pseudoviruses. The rank-order distribution of HIV-1 Env pseudoviruses is shown as described for Fig. 1. Results for individual isolates are color coded based on *k*-means cluster assignments, with the designated tier categories indicated.

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We further examined the robustness of the identified clusters to account for the uncertainty that may be associated with limited sampling (bootstrap) and assay variability (noise). Figure 7 shows a hierarchical clustering heatmap of NAb ID_{50} titers for each virus/plasma pool assay, with HIV-1 Env pseudoviruses in each tier group that clustered with ≥80% probability highlighted in boxes. Viral isolates identified as tier 1A and tier 3 were found to form reproducible clusters when bootstrap and noise were incorporated into the analysis. The majority of isolates identified as tier 1B and tier 2 viruses were also found to cluster consistently; however, there were also a proportion of these viruses that were more ambiguous in group assignment with the addition of these variables. In particular, the results of the analysis suggest that the tier assignment given here is more sensitive to the particular set of pooled sera used for the assignment than to interassay variability (Fig. 7, left hand columns) and that, for some of the Env pseudoviruses (Fig. 7, those outside the rectangles), the classification is less well resolved. Furthermore, since some clades and CRFs are not represented by our panel of sera, the actual uncertainty is likely to have been underestimated by the bootstrap measure. Together, these data demonstrate that four distinct categories of neutralization sensitivity can be identified in this panel of HIV-1 Env pseudoviruses when a complex set of HIV-1^+ plasma pools are employed for NAb assessment.

We created a maximum-likelihood tree indicating the phylogenetic relationships of the gp160 sequences in our Env pseudovirus panel (Fig. 8). The Peruvian clade B viruses had a disproportionately high number of tier 3 viruses relative to the number among the other clade B isolates tested (4 of 7 from Peru versus 2 of 29 others; Fisher's exact test, $P = 0.032$). In particular, 14 tier 3 viruses were sampled from among the 38 Cameroonian CRF02_AG-related, Chinese CRF07_BC, and Peruvian B clade isolates, while only two tier 3 viruses were found among the other 71 isolates (Fisher's exact test, $P = 4.2 \times 10^{-6}$). Given this, it is interesting to note that the clade B plasma pools were comprised of samples from the United States, and there were no Peruvian B or Chinese CRF07_BC plasma samples used in this study. Thus, it is possible that the neutralization resistance of these viruses might have been affected by not having plasma pools that correspond to the locally circulating lineages in Peru and China. Alternatively, locally circulating lineages may have adopted a generally more neutralization-resistant form of the virus. However, CRF02_AG-related viruses from Cameroon were highly resistant to neutralization (tier 3), even when tested with pooled CRF02_AG plasma from Cameroon.

DISCUSSION

We characterized a large and diverse panel of HIV-1 Env pseudoviruses to better define the relative susceptibility of each isolate to NAbs and to identify subgroups of viruses that represent distinct categories of neutralization sensitivity. This panel represents a broad diversity of HIV-1 isolates in terms of genetic subtype, geographic distribution, and stage of infection, including viruses derived from acute HIV-1 infection. Importantly, this virus panel includes previously described reference panels of viruses, as well as isolates representative of clades and regions of the world that are epicenters of the AIDS epidemic. While some specific clades and CRFs are not well represented in this panel (i.e., clades D, G, and F and CRF01_AE), efforts are under way to clone and characterize HIV-1 Env pseudoviruses of these subtypes for utilization in NAb assays. Nonetheless, the existing panel provides a solid diversity of molecularly cloned viruses for characterizing the range of neutralization sensitivities of HIV-1 primary isolates and for defining subgroups of viruses for tiered NAb assessment. Furthermore, the known gp160 sequence of these viruses will facilitate efforts to map the epitope specificities of broadly neutralizing MAbs and sera from vaccinated or infected individuals and will serve as a foundation for future studies to delineate potential neutralization serotypes. Additional detailed information on these isolates and corresponding gp160 sequence data is being made available for reference on the Los Alamos National Laboratories website (www.hiv.lanl.gov/content/nab-reference-strains), and designated reference clones will be made available to other investigators through the NIH ARRRP.

Our initial rank-ordering analysis demonstrates that many isolates in this panel exhibit a similar range of average NAb sensitivities, with a smaller proportion of viruses demonstrating a distinctly more sensitive or resistant phenotype. Most viruses were neutralized by each of the HIV-1^+ plasma pools tested, although the degree of sensitivity to individual pools varied among isolates. The epitope specificities of NAbs represented in the majority of these plasma pools are unknown, although we have previously investigated the constituent samples of the clade B-Zepto and clade C-SA pools (4). These studies found evidence of NAbs targeting the CD4bs of gp120 and, to a lesser extent, the membrane-proximal external region (MPER) of gp41; however, the majority of neutralizing activity could not be effectively mapped. While we did not detect any pronounced synergy or hindrance associated with the pooling of these individual plasma samples in the studies for this report, it should be noted that these experiments were not optimally designed to thoroughly investigate these questions.

Given the range of neutralization sensitivities observed for individual isolates within this panel, we employed clustering analysis as a method to distinguish four subsets of viruses representative of distinct tiers of neutralization sensitivity. The three isolates demonstrating the greatest sensitivity to neutralization (MW965.26, SF162.LS, and MN) form their own unique cluster (tier 1A) and have historically been utilized for tier 1 screening of candidate vaccine sera for minimal NAb activity. Importantly, our analysis has defined an additional subset of viruses with above-average sensitivities that we have termed tier 1B. These isolates will provide an important bridge in the tiered algorithm of testing between the hypersensitive tier 1A isolates and the much larger subgroup of tier 2 HIV-1 isolates that exhibit moderate sensitivity to antibody-mediated neutralization. To date, many candidate HIV-1 vaccine immunogens have been shown to elicit antibody responses that can neutralize tier 1A viruses, yet none have demonstrated the capacity to elicit neutralizing activity against most tier 2 reference viruses (8, 9, 16, 29). Thus, we recommend the inclusion of tier 1B isolates in the screening of candidate vaccine sera as a means to better detect and identify incremental advances in immunogen design that result in NAbs with increasing breadth and potency. The majority of viruses in our panel clustered into

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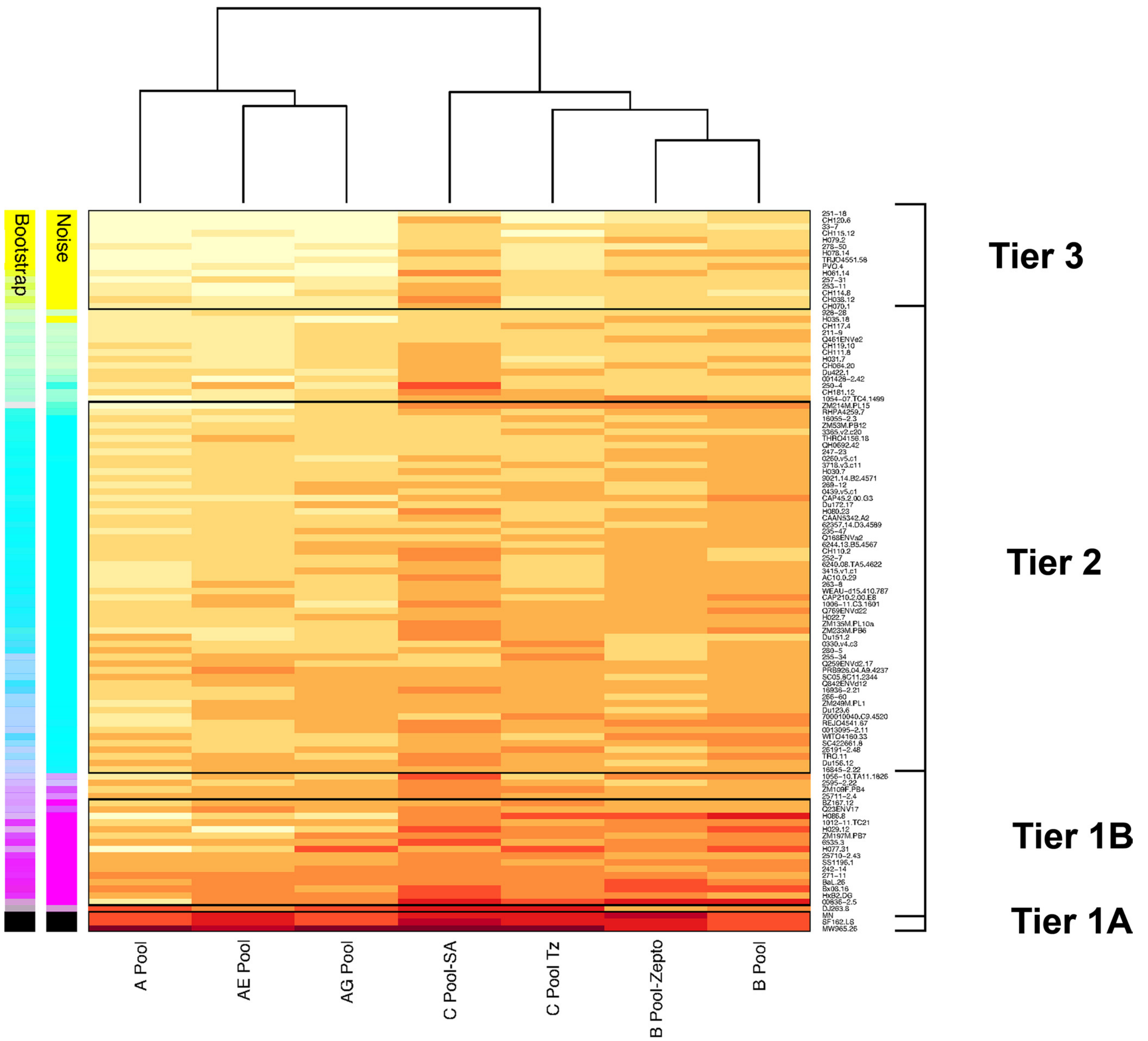

FIG. 7. Hierarchical clustering heatmap demonstrating the robustness of designated tier categorization. HIV-1 Env pseudoviruses ($n = 109$) were assessed for neutralization sensitivities using seven pools of subtype-specific HIV-1^{+} plasma from chronically infected individuals. Individual viruses are listed on the right side based on neutralization sensitivity rank order, and tier categorization as determined by *k*-means clustering analysis is indicated. Individual plasma pools are indicated at the bottom of the heatmap, and the dendrogram for plasma clustering is displayed at the top. The magnitude of neutralization ($\log_{10}$ ID_{50} titers) is denoted by color, in which lower values of neutralization are represented by lighter colors (e.g., light yellows) and higher values are represented by more saturated dark colors (e.g., dark reds). Boxes are drawn around viral isolates in each tier category that grouped with ≥80% probability when uncertainty associated with limited sampling (bootstrap) and assay variability (noise) was incorporated into the *k*-means clustering analysis. The bootstrap involved resampling the data sets from the plasma pools and reevaluating the *k*-means clusters 10,000 times and determining how many times each virus was a member of the originally assigned tier classification in the resampled datasets. Env pseudoviruses that clustered with the assigned tier are shown in the column on the left as black for tier 1A, magenta for tier 1B, blue for tier 2, and yellow for tier 3. The probability of falling within a cluster is indicated by the intensity of the color, and the frequency of falling into each of the respective tiers is indicated by the degree of blending of colors (for example, a virus that consistently grouped with tier 1B would be represented by an intense, solid magenta bar in the left column, while a virus associated with tier 3 in 60% of the bootstraps but with tier 2 in the other 40% would be represented as a faint yellow-green band). A limited number of repeat experiments were performed and used to estimate the impact of interassay variability (called noise), and the repeat error was modeled as a log normal distribution of the results. Noise was added back to the actual data based on randomly selecting points from within the model distribution of error, 1,000 noise-added datasets were generated, and *k*-means clustering was evaluated for each of these. The robustness of the clusters is indicated, in the same manner as the bootstrap, in the second column.

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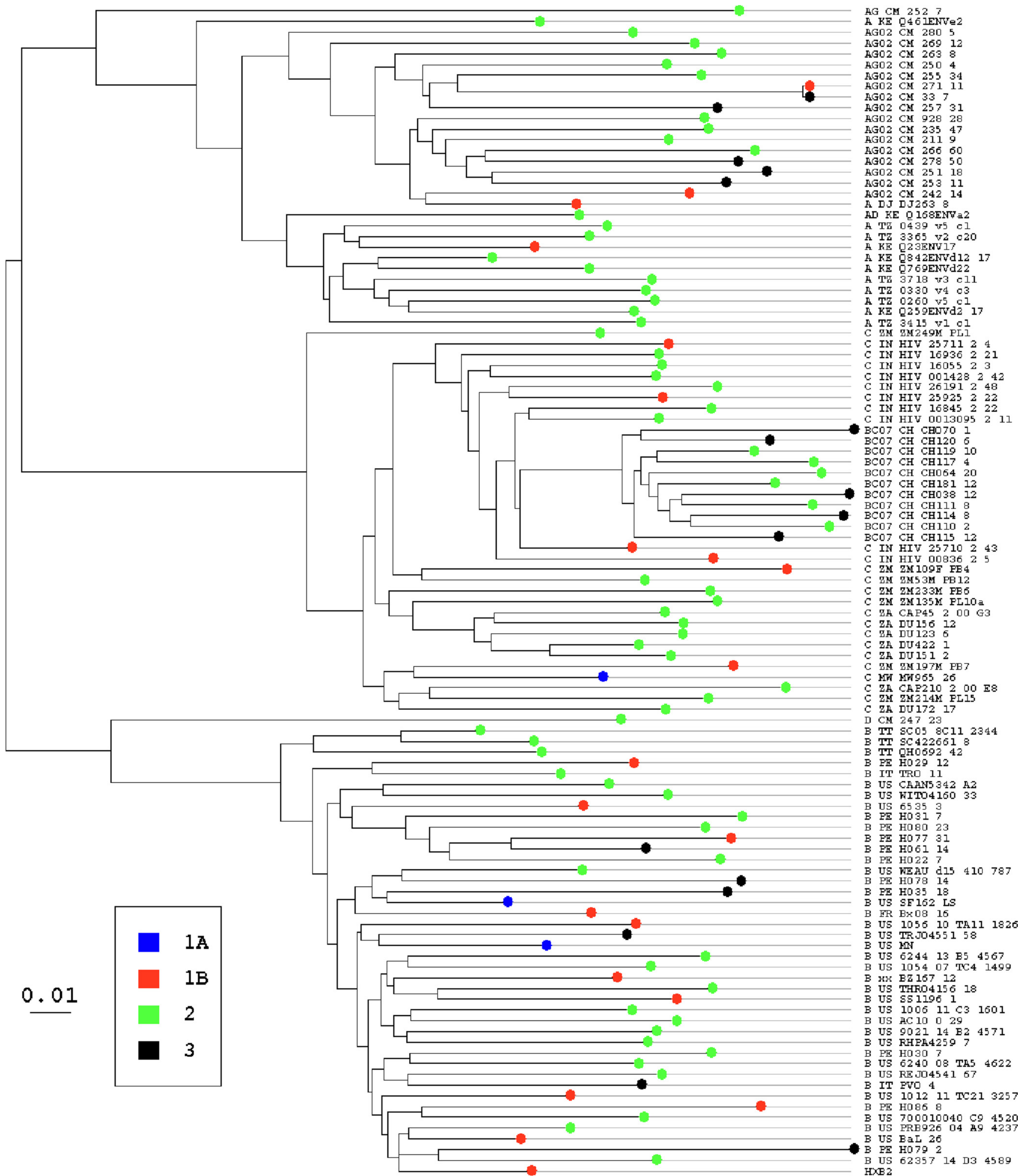

FIG. 8. Phylogenetic tree of Env gp160 sequences. A maximum-likelihood tree indicating the phylogenetic relationships of the Env gp160 sequences from the Env pseudovirus panel is shown. Viruses categorized as tier 1A, 1B, 2, or 3 are color coded as indicated. The scale bar shows the length indicating 1% genetic change.

the tier 2 category and thus may be a fair representation of the average neutralization sensitivity of circulating strains of HIV-1. Utilization of these viruses in the second tier of testing will provide important insight into the potency and cross-reactive breadth of NAbs elicited by candidate vaccine immunogens. We also identified a subgroup of tier 3 viruses that were unusually resistant to neutralization by one or more of the HIV-1^{+} plasma pools. These isolates will provide an additional

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level of stringency for assessing the potency of candidate vaccine sera which demonstrate NAb activity against tier 2 isolates.

We investigated assay parameters in addition to the ID_{50} titer for the rank-ordering and clustering analysis of our HIV-1 Env pseudovirus panel. In particular, pAUC was found to be an informative measure of NAb activity that provided rank order and clustering results that were highly concordant with those of our analyses based on NAb ID_{50} titer. Furthermore, we investigated the robustness of our hierarchical clustering assignments by introducing measures of laboratory assay variance and random resampling with replacement of results for bootstrap comparisons. These analyses demonstrate that the large majority of viruses cluster into their designated tier category with $\geq$80% probability. While particular viruses were ambiguous in tier assignment with the introduction of these variables, the results support the general categorization of these isolates into four tiered subgroups based on neutralization sensitivity. Alternate approaches for identifying the number of clusters (e.g., the method of Calinski and Harabasz [10]) and for assigning viruses to clusters (e.g., *k*-medoids, agglomerative hierarchical clustering, and divisive hierarchical clustering) show some variability in both the number of clusters identified and virus cluster assignments depending on the methods used for analysis. We report the results using *k*-means, a partitional clustering method, but other partitional clustering methods produced very similar results.

The previous studies which led to the recommended standard reference panels of clade B and clade C HIV-1 isolates incorporated NAb phenotyping using four broadly neutralizing MAbs and a limited number of HIV-1^{+} serum samples (22, 23). While these data were useful in selecting viruses which fit the general criteria of not being overly sensitive or resistant to NAbs, it was not clear that these viruses reflected the range of neutralization sensitivities observed in the larger context of globally diverse HIV-1 isolates. In this study, we have applied a more rigorous analytical assessment of the neutralization sensitivities of these commonly used reference viruses. Our results demonstrate that these standard reference panels, as well as several clade A and transmitted/founder clade B viruses, represent a broad distribution in neutralization rank order. While the majority of isolates in these panels were categorized as tier 2 viruses, each panel had one or two viruses that were categorized as tier 1B. The standard clade B reference panel also contains two viruses that were categorized as tier 3 isolates (PVO.4 and TRJO4551.58). Thus, our data support the continued use of these standard reference panels for the testing of candidate vaccine sera. Having defined the relative neutralization sensitivity rank order of viruses within each reference panel will also facilitate the interpretation of results when these panels are employed.

We have also investigated whether specific virus characteristics are associated with neutralization sensitivity. No associations were found with the stage of infection or the source of material from which the gp160 Env gene was cloned. Notably, there was no evidence that the transmitted viruses were different than other viruses in overall neutralization sensitivity. We did observe a significant trend toward increased neutralization sensitivity when the virus and HIV-1^{+} plasma pool were of the same clade, supportive of previous such observations (7). However, it should be noted that this was determined using a limited number of matched virus isolates and plasma pools. To investigate whether this clade match effect influenced our rank ordering and clustering results, these analyses were repeated after the matching clade $\log_{10}$ ID_{50} titers were removed from the average. In fact, we found that exclusion of these data did not greatly affect our results (data not shown).

Some HIV-1-specific MAbs can only neutralize viruses known to have a highly neutralization-sensitive phenotype (5, 9, 34). We therefore evaluated two such MAbs, MAb 1.5E directed to the CD4bs and MAb 17b directed to the coreceptor binding site. Both MAbs could neutralize viruses within the tier 1A and 1B categories but were ineffective against viruses in the tier 2 and 3 categories. Since our virus categorization was done with HIV-1^{+} plasma and not MAbs, these results confirm that the neutralization capacity of some MAbs is restricted to a small subset of particularly neutralization-sensitive viruses. Our data were similar for the anti-V3 MAb 447, which neutralized primarily within the tier 1A and 1B categories of viruses. These data are consistent with a model suggesting that, for most virus isolates, the conformation of the native Env trimer acts to restrict access to coreceptor and CD4 receptor epitopes on gp120 (20, 21, 34) and that the V3 loop region is not well exposed on most primary isolates of HIV-1 (5, 9, 43, 46). Thus, it is possible that the heightened susceptibility of tier 1A and the more sensitive tier 1B viruses to these MAbs and to NAbs in general may be related to an enhanced open configuration of the Env trimer on the virion surface which makes these epitopes more accessible for antibody binding.

In summary, this study provides the first analytically derived definition of tiered neutralization phenotypes of HIV-1 isolates. We used a set of varied plasma pool reagents to characterize the overall neutralization sensitivities of a diverse panel of more than 100 HIV-1 Env pseudoviruses. Viruses were collected from diverse geographic regions and stages of infection and represented most of the major circulating genetic subtypes of HIV-1 worldwide. Viruses could be categorized into four groups, designated tiers 1A, 1B, 2, and 3, with the majority of viruses belonging to tier 2. This viral panel will facilitate the full characterization of novel MAbs and will allow a careful and systematic assessment of the potency and breadth of neutralization by vaccine sera. The known Env sequences and clonal nature of these Env pseudoviruses will also allow investigation of the viral epitopes that contribute to neutralization by HIV^{+} plasma, vaccine sera, and MAbs.

ACKNOWLEDGMENTS

This study was supported by the Bill & Melinda Gates Foundation's Collaboration for AIDS Vaccine Discovery/Comprehensive Antibody Vaccine Immune Monitoring Consortium, grant number 38619, and Vaccine Immunology Statistical Center, grant number 38744.

We thank Kelli M. Greene and Hongmei Gao for their contributions in project management. We thank Lynn Morris (National Institute for Communicable Diseases, Johannesburg, South Africa) and Bruce K. Brown (Henry M. Jackson Foundation, Rockville, MD) for providing plasma samples, James E. Robinson (Tulane University Medical Center, New Orleans, LA) for MAbs 17b and 1.5E, Susan Zolla-Pazner (New York University, New York, NY) for MAb 447-52D, and Sodsai Tovanabutra and Eric Sanders-Buell (Henry M. Jackson Foundation, Rockville, MD) for help in constructing the panel of clade A Envs from Tanzania.

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