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Human Immunodeficiency Virus Type 1 *env* Clones from Acute and Early Subtype B Infections for Standardized Assessments of Vaccine-Elicited Neutralizing Antibodies

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Induction of broadly cross-reactive neutralizing antibodies is a high priority for AIDS vaccine development but one that has proven difficult to be achieved. While most immunogens generate antibodies that neutralize a subset of T-cell-line-adapted strains of human immunodeficiency virus type 1 (HIV-1), none so far have generated a potent, broadly cross-reactive response against primary isolates of the virus. Even small increments in immunogen improvement leading to increases in neutralizing antibody titers and cross-neutralizing activity would accelerate vaccine development; however, a lack of uniformity in target strains used by different investigators to assess cross-neutralization has made the comparison of vaccine-induced antibody responses difficult. Thus, there is an urgent need to establish standard panels of HIV-1 reference strains for wide distribution. To facilitate this, full-length gp160 genes were cloned from acute and early subtype B infections and characterized for use as reference reagents to assess neutralizing antibodies against clade B HIV-1. Individual gp160 clones were screened for infectivity as Env-pseudotyped viruses in a luciferase reporter gene assay in JC53-BL (TZM-bl) cells. Functional *env* clones were sequenced and their neutralization phenotypes characterized by using soluble CD4, monoclonal antibodies, and serum samples from infected individuals and noninfected recipients of a recombinant gp120 vaccine. Env clones from 12 R5 primary HIV-1 isolates were selected that were not unusually sensitive or resistant to neutralization and comprised a wide spectrum of genetic, antigenic, and geographic diversity. These reference reagents will facilitate proficiency testing and other validation efforts aimed at improving assay performance across laboratories and can be used for standardized assessments of vaccine-elicited neutralizing antibodies.

The development of an effective human immunodeficiency virus type 1 (HIV-1) vaccine is believed to require the induction of both virus-specific $CD8^+$ T cells and neutralizing antibodies (Abs) (56, 63). Neutralizing Abs are of particular interest because their presence at the time of intravenous, vaginal, and oral routes of live virus challenge has been shown to prevent AIDS virus infection in nonhuman primates (31, 65, 66, 69, 84, 105). Due to the extraordinary degree of genetic diversity of HIV-1 and the structural complexity of its envelope glycoproteins (Env) (54, 118), designing an effective vaccine is difficult. These same properties of the virus also make it difficult to assess vaccine-elicited neutralizing Abs in a way that is meaningful and informative. Many candidate vaccines have included Env for the purpose of generating an effective neutralizing Ab response, but to date, none appear to elicit a response with the desired specificity and cross-reactivity (5, 6, 13, 68). This poor immunogenicity is incompletely understood but might be explained by either the particular design or low valency of prototype immunogens (14).

A number of new candidate immunogens that aim to overcome the limitations of early prototypes are now being explored. New immunogens include (i) pseudovirions (75), virus-like particles (7, 46, 95), chemically inactivated virus (1, 92), and cleaved and uncleaved gp140 oligomers (8, 26, 52, 98, 120, 121) that aim to mimic native Env structure; (ii) partially deglycosylated Env (10, 88, 89) and variable loop-deleted Env (4, 18, 47, 51, 108) to expose hidden epitopes; (iii) hyperglycosylation to focus the antibody response on key conserved elements of Env (82); (iv) triggered Env structures to stabilize intermediate epitopes formed during binding and fusion (33, 34, 61, 113); (v) structural analogues of conserved epitopes

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recognized by broadly cross-reactive neutralizing Abs (23, 27, 50, 60, 70, 81, 100, 124); and (vi) polyvalent Env, polyvalent peptides, and consensus and ancestral Env to minimize the genetic and antigenic differences between vaccine strains and field isolates (19, 38, 39, 79). Immunogens that incorporate various combinations of these new design concepts are also in development (96, 107).

It is necessary to measure the ability of each new immunogen to elicit high-titer, broadly cross-reactive neutralizing Abs. It is also necessary to obtain standardized measurements of the neutralizing Ab response so that incremental advancements in immunogen design can be identified. Presently, assessments of the neutralizing Ab response involves the use of multiple HIV-1 strains; however, a lack of uniformity in the choice of strains used by different investigators has made it difficult to interpret and compare existing data sets (76). The choice of virus strains used in a neutralizing Ab assay can have a major influence on the results. The inclusion of strains that are either highly sensitive or resistant to neutralization may over- or underestimate the value of the neutralizing Ab response. Additionally, the inclusion of viruses that are genetically or antigenically similar to the vaccine strain makes it difficult to compare neutralizing Ab responses between vaccine studies.

It has been recommended that separate panels of well-characterized reference strains of HIV-1 be developed for each major genetic subtype of the group M viruses (64). The availability and standard use of these reference strains should allow consistent Ab data sets to be acquired and compared. Clade B viruses are the most prevalent genetic subtype of HIV-1 in North America and Europe and were the first viral isolates in the epidemic to be characterized and widely distributed. Thus, many prototype vaccine immunogens have used one or more subtype B viral proteins. An initial panel of reference strains for clade B HIV-1 is described here. This panel comprises 12 well-characterized primary HIV-1 strains that were obtained soon after sexually acquired infection. An infectious full-length gp160 was cloned for each virus. These clones were then cotransfected with an HIV-1 *env*-minus backbone (pSG3Δenv) to generate pseudovirions for use in single-round infection/neutralization assays.

MATERIALS AND METHODS

Serum samples, soluble CD4 (sCD4), and monoclonal antibodies (MAbs). Serum samples were collected from five treatment-naive HIV-1-infected individuals during their routine visits to the Infectious Diseases Clinic at Duke University Medical Center in 2003. These serum samples are most likely representative of clade B infections. Plasma pools were prepared from 6 to 10 subjects with pure clade infections of clades A (pool A), C (pool C), or D (pool D), as verified by full-genome sequencing from DNA obtained from cryopreserved patient peripheral blood mononuclear cells (PBMC). These plasma samples and PBMC were obtained either from anonymous, HIV-positive blood bank units collected in Kenya (clade A) or Tanzania (clade C) or from subjects participating in a study of the molecular epidemiology of HIV infection in Uganda (clade D) (Walter Reed HSRRB log no. A8653; Makere University, Kampala, Uganda assurance no. M-1356). The U.S. HIV-1-positive pool B was prepared from serum samples obtained from subjects participating in a vaccine trial of recombinant gp160 (Walter Reed HSRRB log no. A5392). A normal plasma pool was prepared from leukopaks from four HIV-1-negative subjects (BRT Laboratories, Inc., Baltimore, MD). Serum samples were also collected from participants in a phase I clinical trial, conducted by the HIV Vaccine Trials Network, of a candidate vaccine developed by GlaxoSmithKline Biologicals. Healthy, HIV-1-negative subjects in this trial received a combination vaccine containing HIV-1 NefTat and $gp120_{W61D}$ formulated in a novel adjuvant (AS02A). Serum samples were obtained prior to immunization and 2 weeks after the third inoculation from subjects who received 20 μg NefTat plus 20 μg $gp120_{W61D}$ protein. Additional serum samples were obtained prior to immunization and 2 weeks after the third inoculation from control subjects (adjuvant only). All serum and plasma samples were heat inactivated at 56°C for 1 h prior to use. Informed consent was obtained from all study participants as approved by local institutional review boards and biosafety committees; the blood bank samples were obtained from anonymous donors with all sample codes dislinked from subject identifiers.

Recombinant sCD4 comprising the full-length extracellular domain of human CD4 produced in Chinese hamster ovary cells was obtained from Progenics Pharmaceuticals, Inc. (Tarrytown, NY). Human MAb IgG1b12 was kindly provided by Dennis Burton (The Scripps Research Institute, La Jolla, CA). Human MAbs 2G12, 2F5, and 4E10 were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (NIH ARRRP) as contributed by Hermann Katinger. The V3-specific human MAbs 2182, 2442, 2191, 2219, 2456, and 447D were kindly provided by Susan Zolla-Pazner. TriMab is a mixture of three MAbs (IgG1b12, 2G12, and 2F5) prepared as a 1-mg/ml stock solution containing 333 μg of each MAb/ml in phosphate-buffered saline, pH 7.4.

Cells. JC53-BL cells (also termed TZM-bl cells; catalog no. 8129) were obtained from the NIH ARRRP, as contributed by John Kappes and Xiaoyun Wu. This is a genetically engineered HeLa cell clone that expresses CD4, CXCR4, and CCR5 and contains Tat-responsive reporter genes for firefly luciferase and *Escherichia coli* β-galactosidase under regulatory control of an HIV-1 long terminal repeat (86, 115). 293T cells were obtained from the American Type Culture Collection (catalog no. 11268). Both cell lines were maintained in Dulbecco's modified Eagle's medium (Gibco BRL Life Technologies) containing 10% heat-inactivated fetal bovine serum and 50 μg gentamicin/ml in vented T-75 culture flasks (Corning-Costar). Cultures were incubated at 37°C in a humidified 5% CO_2–95% air environment. Cell monolayers were split 1:10 at confluence by treatment with 0.25% trypsin, 1 mM EDTA (Invitrogen).

Human PBMC were prepared from buffy coats from healthy, HIV-1-negative individuals as obtained from the Laboratory Services of the American Red Cross Carolina Region in Charlotte, NC. PBMC were isolated by centrifugation over lymphocyte separation medium (Organon-Teknika/Akzo, Durham, N.C.). Cells at the interface were washed twice in growth medium (RPMI-1640 containing 20% heat-inactivated fetal bovine serum and 50 μg gentamicin/ml), resuspended at a density of 2.5×10^7 cells/ml, and frozen in 1-ml aliquots in liquid nitrogen with the aid of a Gordonier controlled-rate cryostat. Aliquots of PBMC were thawed in a room-temperature water bath and incubated in growth medium containing phytohemagglutinin (PHA)-P (5 μg/ml) and 4% human interleukin-2 (IL-2) for 1 day at 37°C in a humidified 5% CO_2–95% air environment. The cells were washed and suspended in fresh growth medium containing IL-2 but lacking PHA-P for use in propagating viruses.

Virus strains. T-cell-line-adapted (TCLA) strains $HIV\text{-}1_{IIIB}$ and $HIV\text{-}1_{MN}$ were obtained from Robert Gallo and have been described previously (36). TCLA strain $HIV\text{-}1_{SF\text{-}2}$ (57) was obtained from the NIH ARRRP, as donated by Jay Levy. Cell-free stocks of these viruses were produced in H9 cells as described previously (74). The molecularly cloned pseudovirus SF162.LS has been described previously (17, 109). An assay stock of SF162.LS was prepared by transfection in 293T cells as described below.

Primary isolates for Env cloning were derived from 19 individuals soon after sexually acquired HIV-1 infection. PBMC and plasma samples were obtained in most cases within the first 3 months of infection as determined by either the last known seronegative clinic visit, the time of onset of acute retroviral syndrome, or a combination of these two clinical parameters. An exception is subject 3988, from whom virus was isolated approximately 6 months after HIV-1 infection. Because this subject had no detectable neutralizing Abs at the time of virus isolation (85), it may be assumed that the virus was under no pressure to undergo an initial round of escape. Additional details of clinical studies in which certain subjects were enrolled have been reported for subjects 6101, 5768, 3988, 7165, and 6535 (80); subjects QH0692 and QH0515 (22); subjects SS1196 and BG1168 (103); and subject AC10 (91). A description of the isolation and partial characterization of uncloned primary HIV-1 isolates is available for viruses from subjects 6101, 5768, 3988, 7165, 6535, QH0692, QH0515, SS1196, BG1168, PVO, and TRO (13, 85) and subject AC10 (73). Of note, isolates 6101, 5768, 3988, 7165, and 6535 were previously called P15, P27, P46, P23, and P30, respectively (13, 85).

Amplification and cloning of *rev*/*env* DNA cassettes. *rev*/*env* DNA cassettes containing full-length gp160 were PCR amplified and cloned from either cultured PBMC DNA or plasma HIV-1 RNA. For cloning from DNA, low-passage virus (≤3 passages in PBMC) originating from PBMC coculture was used to inoculate 5×10^7 PHA-stimulated normal donor PBMC at a high multiplicity of infection (>0.01). The medium was replaced with fresh IL-2-containing growth medium every 2 days. Viral p24 Gag antigen synthesis was monitored daily

(Perkin-Elmer Life Sciences) starting on day 4 until the p24 level in culture supernatants was >20 ng/ml. Culture fluids were harvested, filtered, and stored in aliquots as the immediate uncloned parent of the molecularly cloned pseudovirus. Infected cells were collected by centrifugation, washed in sterile phosphate-buffered saline (pH 7.4), and processed for high-molecular-weight DNA extraction using the QIAGEN blood and cell culture DNA kit according to the manufacturer's protocol. PCR amplification was carried out as described previously (37) using primers env1A (5′-CACCGGCTTAGGCATCTCCTATG GCAGGAAGAA-3′) and env1M (5′-TAGCCCTTCCAGTCCCCCCTTTTCT TTTA-3′). Cloning of *rev*/*env* cassettes from plasma viral RNA was carried out as described previously (116). Briefly, virion-associated plasma RNA was purified using the QIAmp viral RNA minikit (QIAGEN) and subjected to cDNA synthesis using SuperScript II (Invitrogen). Viral cDNA was then subjected to nested PCR amplification by using the following primers: outer sense primer 5′-TAGAGCCCTGGAAGCATCCAGGAAG-3′, nucleotides (nt) 5852 to 5876; outer antisense primer 5′-TTGCTACTTGTGATTGCTCCATGT-3′, nt 8912 to 8935; inner sense primer 5′-GATCAAGCTTTAGGCATCTCCTATGGCAGG AAGAAG-3′, nt 5957 to 5982; and inner antisense primer 5′-AGCTGGATCC GTCTCGAGATACTGCTCCCACCC-3′, nt 8881 to 8903. Env clones THRO4156.18 and CAAN5342.A2 were amplified by using the same primers listed above, except for a modified inner sense primer (5′-GATCAAGCTTTA GCCGCCGCCATGGCAGGAAGAAG). Final PCR products were visualized by agarose gel electrophoreses and purified using the QIAquick gel extraction kit (QIAGEN Inc., Valencia, CA). PCR products from PBMC DNA were cloned directly into vector pcDNA 3.1D/V5-His-TOPO (Invitrogen Corp., Carlsbad, CA). This cloning system allows the *env* genes to be inserted in the correct orientation with a cytomegalovirus promoter for protein expression. PCR products from plasma viral RNA were inserted into vector pcDNA 3.1/V5-His-TOPO (Invitrogen Corp., Carlsbad, CA) by TA cloning.

Plasmid minipreps from multiple colonies of transformed JM109 cells were screened by restriction enzyme digestion for full-length inserts. Clones with inserts in the correct orientation were screened for infectivity by cotransfection with an *env*-deficient HIV-1 (SG3Δenv) backbone in 293T cells. Infectivity was determined by titration in JC53-BL cells as described below. Env clones conferring the highest infectivity were selected for further characterization. All newly derived acute/early HIV-1 *env* genes have been donated to the NIH ARRRP (Rockville, MD), and their sequences are available from GenBank and the Los Alamos HIV Sequence Database.

DNA sequence and phylogenetic analysis. Sequence analysis was performed by cycle-sequencing and dye terminator methods with an automated DNA Sequenator (model 3100; Applied Biosystems, Inc.) as recommended by the manufacturer. Individual sequence fragments for each *env* clone were assembled and edited using the Sequencher program 4.2 (Gene Codes Corp., Ann Arbor, MI). Nucleotide and deduced Env amino acid sequences were initially aligned using CLUSTAL W (48, 111) and manually adjusted for an optimal alignment using MASE (29). Pairwise evolutionary distances were estimated using Kimura's two-parameter method (53) to correct for superimposed substitutions; sequence gaps and ambiguous areas within the alignment were excluded from all comparisons. Phylogenetic trees were constructed by the neighbor-joining method (94), and the reliability of branching orders was assessed by bootstrap analysis using 1,000 replicates (30).

Pseudovirus preparation and titration. Pseudovirus was prepared by transfecting exponentially dividing 293T cells (5×10^6 cells in 15 ml growth medium in a T-75 culture flask) with 5 μg of *rev*/*env* expression plasmid and 10 μg of an *env*-deficient HIV-1 backbone vector (pSG3ΔEnv), using PolyFect transfection reagent (QIAGEN). Pseudovirus-containing culture supernatants were harvested 2 days after transfection, filtered (0.45 μm), and stored at −80°C in 1-ml aliquots. The 50% tissue culture infectious dose ($TCID_{50}$) of a single thawed aliquot of each batch of pseudovirus was determined in JC53-BL cells. For $TCID_{50}$ measurements, serial fivefold dilutions of pseudovirus were made in quadruplicate wells in 96-well culture plates in a total volume of 100 μl of growth medium for a total of 11 dilution steps. Freshly trypsinized cells (10,000 cells in 100 μl of growth medium containing 75 μg/ml DEAE-dextran) were added to each well, and the plates were incubated at 37°C in a humidified 5% CO_2–95% air environment. After a 48-hour incubation, 100 μl of culture medium was removed from each well and 100 μl of Bright Glo reagent (Promega Corp., Madison, WI) was added to the cells. After a 2-min incubation at room temperature to allow cell lysis, 150 μl of cell lysate was transferred to 96-well black solid plates (Corning-Costar) for measurements of luminescence using a Victor 2 luminometer (Perkin-Elmer Life Sciences, Shelton, CT). Wells producing relative luminescence units (RLU) >3× background were scored as positive. The $TCID_{50}$ was calculated as described previously (49).

Titration of PBMC-grown primary isolates. $TCID_{50}$ assays were performed exactly as described above for pseudovirions, except that indinavir (NIH ARRRP) was added to the medium at a final concentration of 1 μM to prevent progeny virion production. Indinavir was not needed to titrate pseudovirions because these viruses are replication-defective by design.

Analysis of coreceptor usage. Coreceptor usage of the newly derived Envs was determined using a modification of the JC53-BL infectivity assay and two blocking agents: AMD 3100, which specifically blocks the CXCR4 receptor, and TAK-779, which inhibits binding to the CCR5 receptor (106, 122). JC53-BL cells were seeded overnight and treated for 1 h with AMD 3100 (1.3 μM/well), TAK-779 (10 μM/well), or a combination of the two chemokine receptor-blocking agents. One thousand infectious units of pseudotyped virions was added to each well in the presence of 30 μg/ml DEAE-dextran and incubated at 37°C for 4 h. Following a 2-day incubation, cells were lysed and luciferase units determined. Wells containing the coreceptor inhibitor were compared to control wells containing no inhibitor to determine if either agent led to a reduction in infectivity. YU-2, a virus known to use the CCR5 coreceptor, and NL4.3, a dualtropic virus, were included as controls.

Neutralizing Ab assay. Neutralizing Abs were measured as reductions in Luc reporter gene expression after a single round of virus infection in JC53-BL cells as described previously (72). This assay is a modified version of the assay used by Wei et al. (116). Briefly, 200 $TCID_{50}$ of virus was incubated with various dilutions of test samples (eight dilutions, threefold stepwise) in triplicate for 1 h at 37°C in a total volume of 150 μl growth medium in 96-well flat-bottom culture plates (Corning-Costar). Freshly trypsinized cells (10,000 cells in 100 μl of growth medium containing 75 μg/ml DEAE-dextran) were added to each well. Indinavir was added at a final concentration of 1 μM in cases where uncloned, replication-competent viruses were used. One set of eight control wells received cells plus virus (virus control), and another set of eight wells received cells only (background control). After a 48-h incubation, 150 μl of culture medium was removed from each well and 100 μl of Bright Glo reagent was added to the cells. After a 2-min incubation at room temperature to allow cell lysis, 150 μl of cell lysate was transferred to 96-well black solid plates for measurements of luminescence using a Victor 2 luminometer. The 50% inhibitory dose (ID_{50}) was defined as either the serum dilution or sample concentration (in the case of sCD4 and MAbs) that caused a 50% reduction in RLU compared to virus control wells after subtraction of background RLU. In some cases, serum samples were screened for neutralizing activity at a single dilution. Briefly, serum samples obtained before and after immunization from vaccinated subjects were tested in parallel at a 1:10 dilution in triplicate. The percent reduction in RLU was calculated relative to the RLU in the presence of preimmune serum.

A modified version of the JC53-BL neutralizing Ab assay was used to evaluate V3-specific MAbs in a separate laboratory (that of L. Stamatatos). The assay used JC53-BL cells that were seeded at a density of 3,000 cells/well in 96-well culture plates and allowed to adhere overnight before addition of serially diluted MAbs that had been mixed with virus (1.5-h incubation of MAbs plus virus). JC53-BL cells were treated for 30 min with medium containing Polybrene (2 ng/ml) and washed with fresh growth medium immediately before addition of the MAb-virus mixtures. Luminescence was measured 72 h later. A preliminary unblinded comparison of the two assay formats, using 2F5 and 13 different Env-pseudotyped strains of HIV-1, demonstrated a high level of concordance (data not shown).

Statistical analysis. Wilcoxon signed-rank tests were used to assess if neutralization titers against viruses differed between HIV-1-positive plasma pools. For each of the 19 molecularly cloned pseudoviruses, Wilcoxon rank sum tests were used to assess if neutralization titers differed between vaccine recipients and control subjects. All *P* values were adjusted using the Bonferonni method to account for the large number of tests, and adjusted *P* values of <0.05 indicated statistical significance.

Nucleotide sequence accession numbers. GenBank accession numbers for the sequences determined in this study are AY835434 to AY835453.

RESULTS

Cloning of full-length HIV-1 *env* genes. Full-length *env* clones (*rev*/*env* cassettes) were cloned from short-term-cultured PBMC DNA of 12 primary isolates obtained from patients with acute or early HIV-1 infection (Table 1; Fig. 1). Corresponding culture supernatants from the infected PBMC were harvested, filtered, and stored in aliquots as reference stocks of the immediate uncloned parental viruses (Fig. 1).

FIG. 1. Generation of pseudovirions containing cloned Env from primary PBMC-cultured isolates. Primary isolates of HIV-1, obtained by standard PBMC coculture, were used to infect fresh cultures of PHA-stimulated PBMC. DNA from the infected PBMC was used for the PCR amplification and cloning of *rev*/*env* cassettes that contained full-length gp160 genes. The virus-containing culture medium was made cell free by 0.45-μm filtration and stored in aliquots at −80°C as a reference stock of the immediate uncloned parental virus. Cloned gp160 genes were used to produce Env-pseudotyped virus in 293T cells by cotransfection with the pSG3ΔEnv backbone vector. Neutralization phenotypes of the parental PBMC viruses and pseudovirions generated by cotransfection with the cloned primary Envs were compared in JC53-BL cells.

TABLE 1. Demographic and biologic properties of molecularly cloned, Env-pseudotyped strains of clade B HIV-1

Env clone[a]	Panel designation[a]	Coreceptor	Mode of transmission[b]	Date isolated	Last known seronegative date	Approximate length of time of infection (wk)	Location	Plasma viral load (copies/ml)	CD4 count	Source	Accession no.
6101.10	SVPB1	R5	M-M	09/19/94	08/25/94	8	Washington, D.C.	744,000	704	ccPBMC[c]	AY835434
5768.4	SVPB2	R5	M-M	04/19/95	03/25/95	8	Washington, D.C.	40,000	441	ccPBMC	AY835435
3988.25	SVPB3	R5	M-M	08/31/95	NA[d]	24	Washington, D.C.	43,000	848	ccPBMC	AY835436
7165.18	SVPB4	R5	M-M	12/12/94	10/28/94	6	Washington, D.C.	171,000	756	ccPBMC	AY835437
6535.3*	SVPB5*	R5	M-M	05/10/95	03/24/95	6	Washington, D.C.	1,224,000	558	ccPBMC	AY835438
QH0692.42*	SVPB6*	R5	F-M	12/01/94	10/24/94	6	Trinidad	9,611	NA	ccPBMC	AY835439
QH0515.1	SVPB7	R5	F-M	08/15/94	07/25/94	4	Trinidad	4,563,504	NA	ccPBMC	AY835440
SC422661.8*	SVPB8*	R5	F-M	01/18/95	12/12/94	4	Trinidad	1,380,000	NA	Plasma	AY835441
SS1196.1	SVPB9	R5	M-M	02/97	07/02/96	16	Washington	NA	NA	ccPBMC	AY835442
BG1168.1	SVPB10	R5	M-M	07/96	02/02/96	4	Washington	NA	NA	ccPBMC	AY835443
PVO.4*	SVPB11*	R5	M-M	01/12/96	NA	4	Italy	2,127,976	311	ccPBMC	AY835444
TRO.11*	SVPB12*	R5	M-M	12/12/95	NA	4	Italy	105,190	335	ccPBMC	AY835445
AC10.0.29*	SVPB13*	R5	M-M	03/19/98	NA	4	Massachusetts	40,700	919	ccPBMC	AY835446
RHPA4259.7*	SVPB14*	R5	M-F	12/05/00	NA	<8	Tennessee	1,458,354	247	Plasma	AY835447
THRO4156.18*	SVPB15*	R5	M-M	08/01/00	07/23/00	1	Alabama	5,413,140	289	Plasma	AY835448
REJO4541.67*	SVPB16*	R5	F-M	09/28/01	09/12/01	2	Alabama	722,349	848	Plasma	AY835449
TRJO4551.58*	SVPB17*	R5	M-M	10/10/01	10/06/01	1	Alabama	8,122,951	NA	Plasma	AY835450
WITO4160.33*	SVPB18*	R5	F-M	08/04/00	08/07/00	1	Alabama	325,064	253	Plasma	AY835451
CAAN5342.A2*	SVPB19*	R5	M-M	04/13/04	NA	<12	Alabama	>1,000,000	278	Plasma	AY835452

[a] Clones selected as standard reference strains are marked with an asterisk.
[b] M, male; F, female.
[c] ccPBMC, cocultured PBMC.
[d] NA, not available.

rev/*env* cassettes from seven additional acutely infected subjects were cloned directly from plasma viral RNA. Sequence analysis of multiple plasma-derived *env* clones (9 to 12 clones per patient) revealed a homogeneous virus population for all seven subjects, with average V1V5 nucleotide sequence differences of less than 1.2% (not shown). Multiple clones from both the PBMC viruses and plasma viruses were screened for infectivity in JC53-BL cells as Env-pseudotyped viruses, and representative clones were selected for further analysis. Clones with the highest infectivity were selected for the technical advantage of generating high-titer stocks of Env-pseudotyped viruses for neutralization assays. Multiple clones of each plasma-derived *env* were screened in neutralization assays with high-reactive and low-reactive plasma pools from HIV-1-infected individuals. Uniform results were obtained with all clones of a corresponding *env* in most cases, although we did occasionally detect a single outlier clone that was unusually sensitive compared to other clones in the matched set. These outlier clones were not selected for further use in this study. All selected clones used CCR5, but not CXCR4, to gain entry into JC53-BL cells.

Acute/early infections occurred between 1994 and 2004 in the United States, Italy, and Trinidad. Thirteen infections arose from male-male transmission, five from female-male transmission, and one from male-female transmission. Plasma viral loads at the time of virus isolation in many subjects were typical of the high levels seen at or near the peak of viremia soon after primary infection (21, 24, 103). Lower levels of plasma viral RNA in other cases were an indication that some viruses may have been isolated either prior to or after the initial peak in plasma viremia. It was possible to document in 13 cases that viruses were obtained within 6 weeks from the time of seroconversion. No serologic testing was performed for the remaining eight subjects prior to seroconversion. For these

remaining subjects, the duration of infection from the time of onset of symptoms of acute retroviral infection (102) was estimated to be 24 weeks (3988.25), 4 weeks (BG1168.1, PVO.4 and TRO.11), 6 weeks (RHPA4259.7), and < 3 months (CAAN.A2). Subject AC10 was seropositive at the time of virus isolation, but Western blot reactivity indicated a very early stage of seroconversion (73). Subject SS1196 had no symptoms of acute infection, and therefore the duration of infection at the time of virus isolation (<4 months) was taken as the midpoint between the last negative serologic test and the first positive serologic test.

Sequence analysis of full-length HIV-1 *env* genes. Phylogenetic analysis of full-length gp160 nucleotide sequences confirmed that all 19 functional clones grouped within clade B (Fig. 2). A wide spectrum of genetic diversity was readily apparent between most clones, as would be expected for independent infections. Only two clones, both from Trinidad (SC422661.8 and QH0515.1), clustered with a high bootstrap value. This linkage is consistent with a previous report on these and other isolates from Trinidad (22). Deduced amino acid sequences showed an uninterrupted *env* open reading frame for 18 of the 19 functional gp160 clones (Fig. 3). Clone 6101.10 contained a premature stop codon that is predicted to truncate the terminal 100 amino acids of the cytoplasmic domain of gp41.

Natural variation in the native structure of Env trimeric spikes on the HIV-1 virion is thought to influence the neutralization phenotype of the virus. In particular, epitope exposure may be influenced in part by the size and structure of variable loops (11, 28, 77) and the position of N-linked glycosylation sites (67, 89, 116). Cysteine residues that form the V1/V2, V3, and V4 loops of gp120 were conserved in all 19 clones (Fig. 3). Clone BG1168.1 contained two additional cysteine residues in V1. Another clone (PVO.4) contained an extra cysteine pair in the central portion of V4. Most size variation was seen in the V1/V2 and V5 loops and was most pronounced in the central region of V1 and the C-terminal region of V2 (Table 2). Little size variation was seen in V3 and V4.

Moderate variation was seen in the number and position of potential N-linked glycosylation sites in gp120, ranging in number from 22 to 27 sites (Table 3) and most heavily clustered in V1/V2 and V4 (Fig. 3). Five sites were conserved on all 19 clones, including one site at the N terminus of the V3 loop that has been shown to mask adjacent neutralization epitopes (2, 104). All clones also contained four highly conserved potential N-linked glycosylation sites in the gp41 ectodomain. Two clones contained a fifth potential N-linked glycosylation site in the membrane-proximal ectodomain of gp41.

Neutralization phenotypes. One of our goals in devising a standard panel of clade B *env* reference clones was to select clones that are not unusually sensitive or resistant to neutralization while at the same time preserving an ability to detect multiple antibody specificities that are not biased toward a particular antibody repertoire. Because the antibody specificities that confer protection against HIV-1 are unknown, a wide spectrum of serologic reagents was used to characterize neutralization phenotypes. Individual serum samples and pooled plasma samples from HIV-1-infected individuals were used as a means to evaluate epitopes targeted during infection. Sensitivity to inhibition by sCD4 was used as a relative measure of epitope exposure in and around the CD4 binding site of gp120.

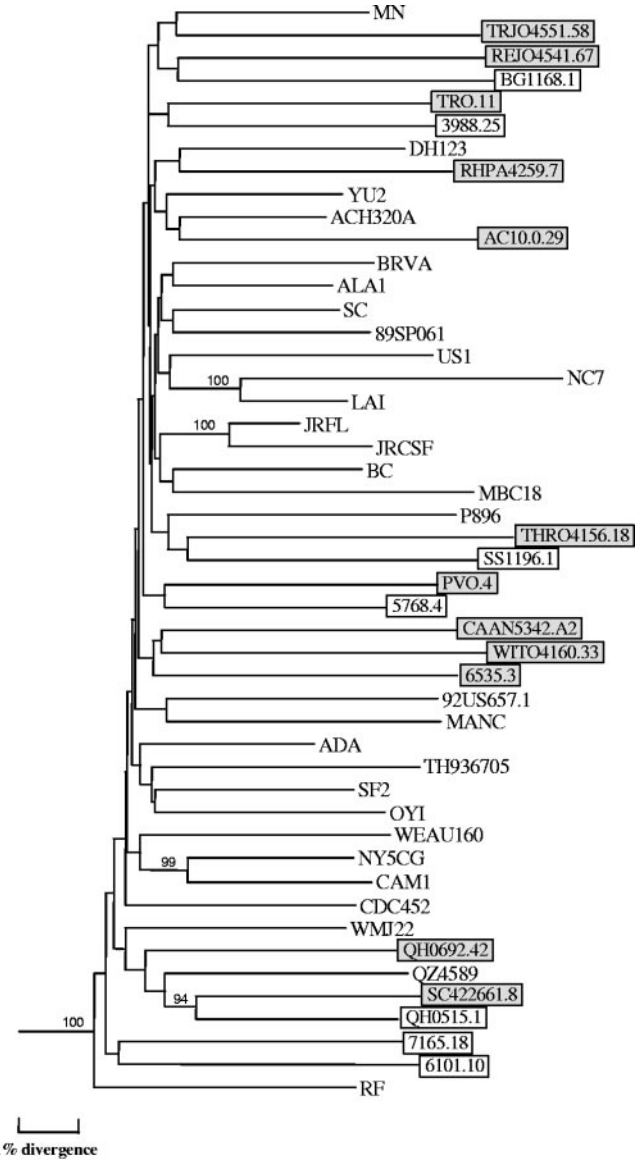

FIG. 2. Phylogenetic relationships of newly derived HIV-1 *env* sequences with subtype B reference strains. The newly characterized sequences are indicated by boxes, and the 12 *env* clones that are recommended as standardized reference reagents are highlighted by shaded boxes. Horizontal branch lengths are drawn to scale (the scale bar represents 0.01 nucleotide substitution per site), but vertical separation is for clarity only. Values at nodes indicate the percentage of bootstraps in which the cluster to the right was found; only values of 80% or greater are shown. The phylogenetic tree was rooted with subtype D *env* sequences (NDK, Z2Z3, and 94UG114).

Four human MAbs with broadly cross-reactive neutralizing activity were used, because many novel immunogens aim to generate antibodies with similar specificities. Three of these MAbs were used in combination (TriMab) as a possible broad-spectrum positive control reagent. Additional MAbs were used to probe neutralization epitopes in the V3 loop.

The results of a comprehensive analysis of the neutralization phenotypes of the 19 pseudoviruses containing cloned acute/early infection Env are shown in Table 4. We included MN,

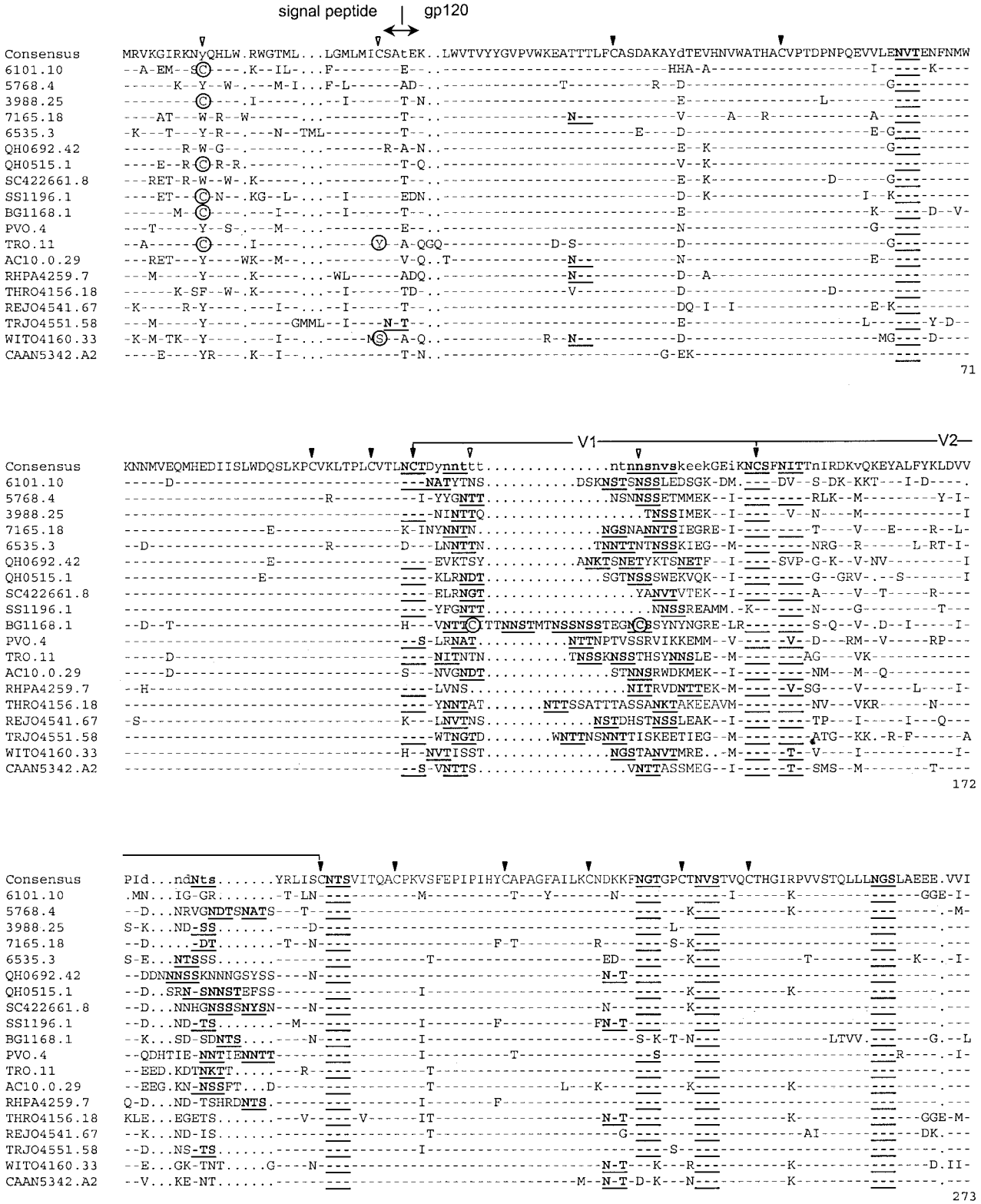

FIG. 3. Alignment of deduced amino acid sequences from acute/early HIV-1 *env* genes. Nucleotide sequences of newly derived *env* genes were translated, aligned, and compared with a consensus sequence generated by MASE. Numbering of amino acid residues begins with the first residue of gp120 and does not include the signal peptide. Dashes indicate sequence identity, while dots represent gaps introduced to optimize alignments. Lowercase letters in the consensus sequence indicate sites at which fewer than 50% of the viruses share the same amino acid residue. Triangles above the consensus sequence indicate cysteine residues (solid triangles indicate sequence identity, while open triangles indicate sequence variation). V1, V2, V3, V4, and V5 regions designate hypervariable HIV-1 gp120 domains as previously described. The signal peptide and Env precursor cleavage sites are indicated. msd, membrane-spanning domain in gp41; asterisks, in-frame stop codons; open circles, altered cysteine residues in the extracellular portions of gp120 and gp41. Potential N-linked glycosylation sites (NXYX motif, where X is any amino acid other than proline and Y is either serine or threonine) are in boldface and underlined.

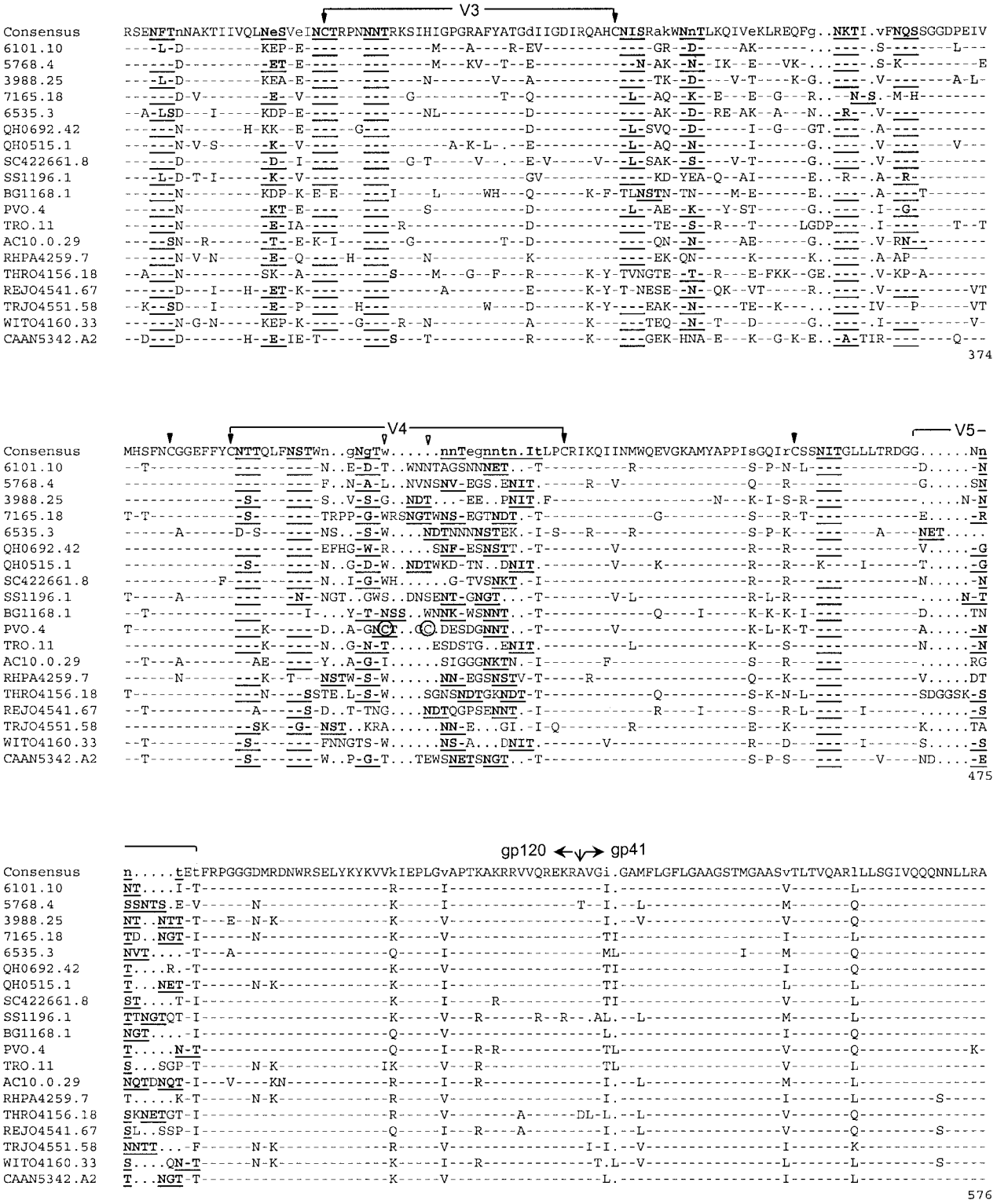

FIG. 3—*Continued*.

IIIB, and SF162.LS in this analysis to allow comparison to strains that are known to be highly sensitive to neutralization. The acute/early Env-pseudotyped viruses exhibited variable patterns of neutralization by individual patient serum samples and pooled plasma samples. Most of these Env-pseudotyped viruses were relatively insensitive to neutralization by individual serum samples from four HIV-1-infected donors compared to the high sensitivity exhibited by MN, IIIB, and SF162.LS. A

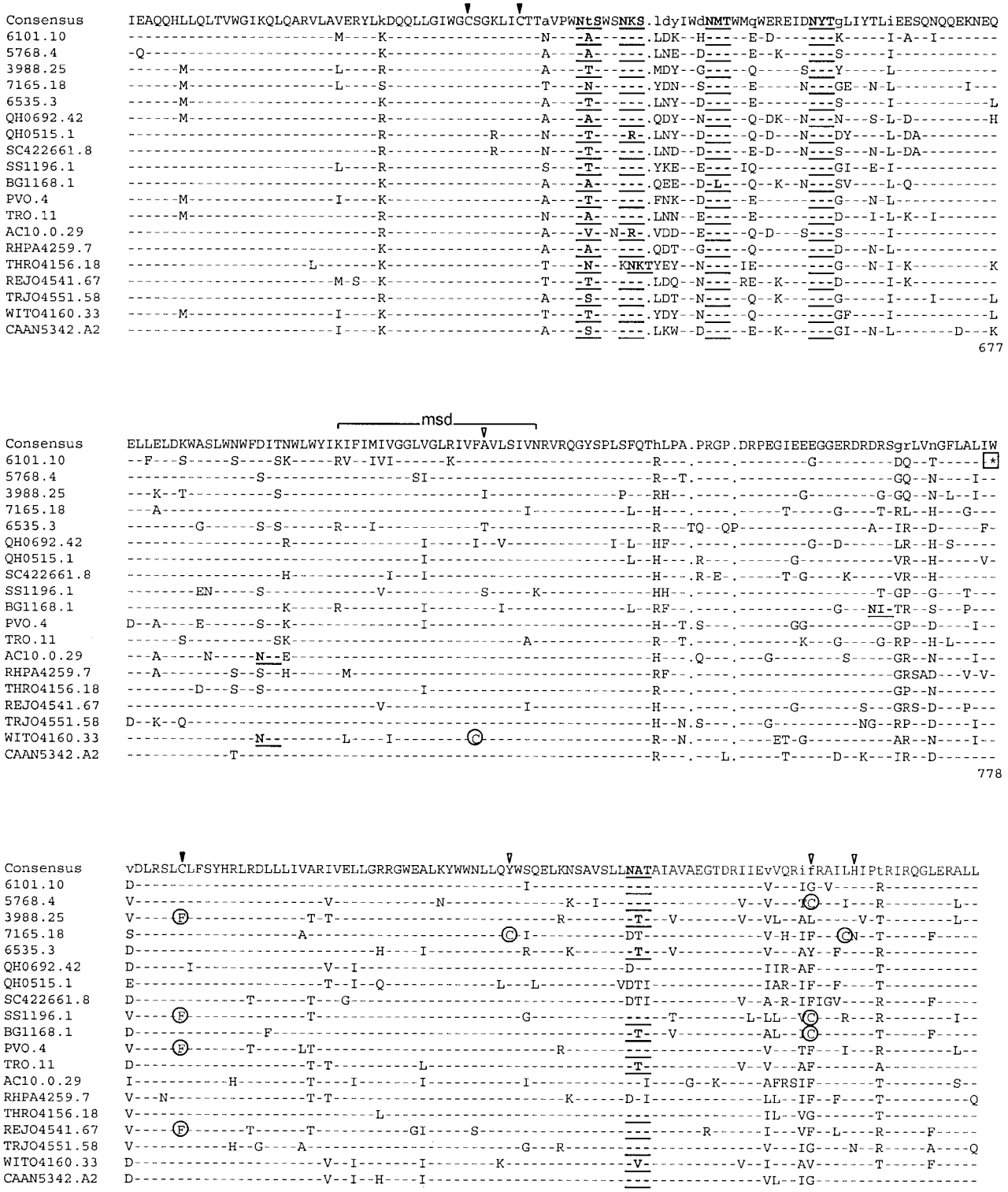

FIG. 3—*Continued*.

fifth HIV-1-positive serum sample, DUMC-1, exhibited potent neutralizing activity against all viruses, which is an unusual property for serum from a single donor. We have found that this serum sample has potent neutralizing activity against a broad spectrum of additional HIV-1 strains regardless of genetic subtype (unpublished data).

Pseudoviruses containing acute/early Env were broadly sensitive to neutralization by HIV-1-positive plasma pools repre-

TABLE 2. Lengths of variable loops

Env clone[a]	No. of amino acids in:			
	V1/V2	V3	V4	V5
6101.10	67	35	34	9
5768.4	73	35	34	12
3988.25	62	35	30	11
7165.18	66	35	39	11
6535.3*	68	35	33	9
QH0692.42*	80	35	34	8
QH0515.1	74	34	33	10
SC422661.8*	70	34	31	9
SS1196.1	60	35	33	13
BG1168.1	80	35	33	11
PVO.4*	81	35	33	8
TRO.11*	74	35	30	10
AC10.0.29*	72	35	31	12
RHPA4259.7*	70	35	34	8
THRO4156.18*	74	35	35	18
REJO4541.67*	68	35	33	10
TRJO4551.58*	73	35	28	10
WITO4160.33*	66	35	30	9
CAAN5342.A2	64	35	33	11
Mean	70.6	34.9	32.7	10.5

[a] Clones selected as standard reference strains are marked with an asterisk.

senting clades A through D. The overall potencies of the plasma pools ranked in order clade C > clade B > clade A > clade D. Differences in potency were significant for the clade C pool compared to clade A ($P = < 0.001$) and D ($P = < 0.001$) pools. Differences in potency also were significant for the clade B pool compared to the clade A ($P = 0.004$) and D ($P = < 0.001$) pools. No significant differences in potency were seen between the clade B and C pools ($P = 0.06$) and the clade A and D pools ($P = 1.0$). These results suggest that clade B viruses are more sensitive to neutralization by plasma from individuals who are infected with clade B and C viruses compared to infection with clade A and D viruses.

TABLE 3. Numbers of potential N-linked glycosylation sites in gp120 and gp41

Env clone[a]	No. of potential N-linked glycosylation sites in:		
	gp120	gp41	gp41 ectodomain
6101.10	22	4	4
5768.4	26	5	4
3988.25	25	5	4
7165.18	27	4	4
6535.3*	25	5	4
QH0692.42*	26	4	4
QH0515.1	27	4	4
SC422661.8*	26	4	4
SS1196.1	24	5	4
BG1168.1	24	6	4
PVO.4*	27	5	4
TRO.11*	27	5	4
AC10.0.29*	24	5	5
RHPA4259.7*	24	4	4
THRO4156.18*	25	5	4
REJO4541.67*	22	5	4
TRJO4551.58*	26	5	4
WITO4160.33*	25	6	5
CAAN5342.A2*	23	5	4
Mean	25.0	4.8	4.1

[a] Clones selected as standard reference strains are marked with an asterisk.

The geometric mean titer (GMT) of neutralizing activity exhibited by HIV-1-positive serum samples and plasma pools was calculated for each Env-pseudotyped virus and used to rank the clones in order of neutralization sensitivity (Fig. 4). Acute/early Env-containing pseudoviruses were considerably less sensitive to neutralization than MN, SF162.LS, and, to a lesser extent, IIIB in a majority of cases. Specifically, these pseudoviruses were 50- to 500-fold less sensitive to neutralization than were MN and SF162.LS and were 5- to 50-fold less sensitive than IIIB. The neutralization phenotypes of two Env-pseudotyped viruses (SS1196.1 and TRJO4551.58) closely resembled that of IIIB.

All acute/early Env-pseudotyped viruses were sensitive to sCD4, but, in general, they were much less sensitive than MN, IIIB, and SF162.LS. Specifically, they were 30-fold to 1,770-fold less sensitive than HIV-1 MN and IIIB (mean, 370-fold) and 6-fold to 354-fold less sensitive than SF162.LS (mean, 74-fold).

As expected, all pseudoviruses carrying acute/early Env were neutralized by one or more human MAbs. The broadest sensitivity was seen with IgG1b12 and/or 2G12. IgG1b12 recognizes a complex epitope in the CD4-binding domain of gp120 that is sensitive to mutations in V2 and C3 (15, 71, 81). 2G12 recognizes a mannose cluster on gp120 that can involve N-linked glycans at multiple sites, including residues 295, 332, and 392, with possible contributions from residues 339 and 386 (9, 16, 97, 101). Sequence motifs predicting N-linked glycosylation at all of these residues were conserved in 11 Env clones, only one of which (clone TRJO4551.58) was resistant to 2G12 (Fig. 3). Two additional clones lacked predicted N-linked glycosylation sites at residues 339 (clone SS1196.1) and 386 (clone 6535.3) but remained sensitive to 2G12, confirming that these two residues are not always essential for 2G12 recognition. The remaining six clones lacked one or more predicted N-linked glycosylation sites at residues 295, 332, 339, 386, and 392 and were resistant to 2G12. In two cases, 2G12 resistance was associated with a loss of predicted N-linked glycosylation at either residue 295 (clone AC10.0.29) or 332 (clone THRO4156.18).

Thirteen acute/early Env-containing pseudoviruses were sensitive to neutralization by 2F5. The epitope for this MAb resides in the membrane-proximal ectodomain of gp41 and involves residues ELDKWAS (3, 77, 78, 87), with possible contributions from flanking residues (83). In a recent study of 90 HIV-1 strains belonging to multiple genetic subtypes, the DKW motif proved to be the minimum requirement for 2F5 recognition (9). Our results are in general agreement with this observation. Thus, 11 of 13 Env clones containing the DKW motif were sensitive to neutralization by 2F5. Two additional Env clones (PVO.4 and THRO4156.18) contained this motif and were not sensitive to 2F5; however, both of these Env clones contained sequence changes elsewhere in the 2F5 epitope. The remaining 2F5-resistant Env clones contained a mutation in the DXW motif.

All 19 acute/early Env-pseudotyped viruses were highly sensitive to neutralization by 4E10. This MAb targets an epitope (NWFDIT) located immediately downstream from the 2F5 epitope in the membrane-proximal ectodomain of gp41 (110, 125). Recent results suggest that the WFXI motif is the minimum requirement for 4E10 neutralization (9). In agreement

TABLE 4. Neutralization phenotypes of pseudoviruses containing cloned Env from acute or early HIV-1 infections as determined with serum from HIV-1-infected individuals, sCD4, and MAbs.

Virus[a]	Panel designation[a]	ID_{50} in TZM-bl cells[b]															
		Reciprocal serum dilution										μg/ml					
		DUMC-1	DUMC-2	DUMC-3	LW.0013	TH.10.03	Pool A	Pool B	Pool C	Pool D	Normal pool	sCD4	IgG1b12	2G12	2F5	4E10	TriMab
MN		15,260	8,083	1,541	5,972	11,325	14,095	43,264	6,253	10,181	<20	0.01	0.01	>50	0.01	0.01	0.01
SF162.LS		>43,740	43,735	1,178	9,756	41,073	6,305	>43,740	25,818	3,442	25	0.05	0.01	0.6	0.1	0.3	0.03
IIIB		2,861	744	69	1,873	265	343	803	547	757	<20	0.01	0.01	1.0	0.2	0.2	0.04
6101.10	SVPB1	1,636	<20	<20	27	<20	41	87	161	38	<20	6.0	>50	3.0	>50	0.3	10.1
5768.4	SVPB2	488	<20	<20	40	53	53	209	354	122	<20	4.0	3.0	9.0	12	6.1	0.9
3988.25	SVPB3	1,729	29	97	154	133	426	313	477	104	45	12.0	0.4	0.3	>50	0.5	0.8
7165.18	SVPB4	107	<20	<20	<20	22	179	119	356	95	60	4.3	>50	0.9	3.9	1.1	1.2
6535.3*	SVPB5*	409	337	<20	118	82	273	478	465	61	<20	0.8	1.4	2.0	1.9	0.2	1.2
QH0692.42*	SVPB6*	462	40	68	54	53	85	374	237	115	105	0.5	0.3	2.8	1.0	1.4	0.3
QH0515.1	SVPB7	874	<20	<20	<20	<20	<20	47	125	40	<20	8.0	2.6	0.1	10	5.0	0.1
SC422661.8*	SVPB8*	5,148	<20	51	235	113	63	248	317	167	96	4.7	0.2	2.1	0.7	0.9	0.3
SS1196.1	SVPB9	3,916	254	80	177	129	346	2,457	1,742	306	48	0.8	0.3	12.0	24	0.3	0.5
BG1168.1	SVPB10	305	<20	21	<20	<20	58	58	179	46	26	10.0	>50	>50	0.8	1.8	3.5
PVO.4*	SVPB11*	2,497	<20	50	<20	39	50	105	534	57	57	6.7	>50	1.2	>50	6.5	4.2
TRO.11*	SVPB12*	4,571	<20	<20	131	<20	298	282	750	168	28	11.5	>50	0.4	>50	0.3	0.8
AC10.0.29*	SVPB13*	89	<20	<20	81	31	90	104	235	40	29	8.5	1.9	>50	1.3	0.3	0.9
RHPA4259.7*	SVPB14*	1,473	<20	30	94	78	87	188	455	117	<20	1.8	0.1	>50	12.0	6.9	0.1
THRO4156.18*	SVPB15*	330	71	37	147	66	69	114	367	55	<20	0.3	0.5	>50	>50	0.3	1.0
REJO4541.67*	SVPB16*	3,634	290	54	541	227	258	503	608	465	<20	0.5	0.7	>50	0.6	0.7	0.5
TRJO4551.58*	SVPB17*	570	<20	<20	147	22	97	119	383	97	<20	20.2	>50	>50	>50	4.5	>25
WITO4160.33*	SVPB18*	4,410	<20	<20	73	34	280	214	376	216	<20	5.4	3.1	1.1	0.6	0.3	0.7
CAAN5342.A2*	SVPB19*	423	<20	21	77	51	143	105	215	73	<20	16.0	>50	>50	3.6	2.7	17.9
GMT[c]		936	21	22	65	41	107	189	361	97	21	3.6				1.0	0.9

[a] Clones selected as standard reference strains are marked with an asterisk.

[b] Values are the dilution or concentration at which RLU were reduced 50% compared to those in virus control wells. DUMC-1, DUMC-2, DUMC-3, LW.0013, and TH.10.03 are serum samples from individuals infected with clade B HIV-1.

[c] Geometric mean titer against the 19 pseudoviruses containing cloned primary isolate Env (excludes MN, SF162.LS, and IIIB). Serum/plasma titers of < 20 were assigned a value of 10 for calculations. The TriMab ID_{50} of > 25 against TRJO4551.58 was assigned a value of 50. Due to multiple ID_{50} values of > 50, the GMT was not determined for IgG1b12, 2G12, and 2F5.

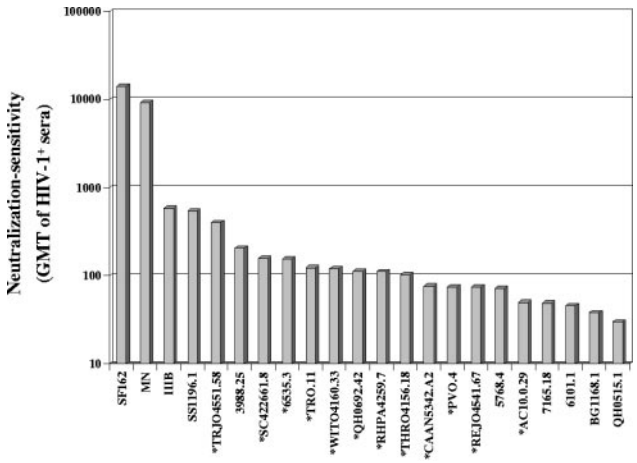

FIG. 4. Rank order of the neutralization sensitivities of acute/early HIV-1 Env clones as determined with serum and plasma samples from HIV-1-infected individuals. The bar height represents the GMT of the HIV-1-positive serum and plasma samples shown in Table 4 (samples DUMC-1, DUMC-2, DUMC-3, LW.0013, and TH.10.3 and pool A, pool B, pool C, and pool D). Clones selected as standard reference strains are marked with an asterisk.

with this observation, D→S was the most common sequence change in the 4E10 epitope of the Env clones examined here (Fig. 3). Notably, two clones (AC10.0.29 and WITO4160.33) contained a potential N-linked glycosylation site (D→N) that would be expected to shield the virus from 4E10 recognition. The fact that both Env clones were sensitive to 4E10 suggests an absence of glycans at this site. Biochemical evidence for a lack of glycosylation at this site has been reported previously (55).

TriMab exhibited potent neutralizing activity (ID_{50} of <6 μg/ml) against all acute/early Env-pseudotyped viruses except TRJO4551.58, which resisted neutralization (ID_{50} of >25 μg/ml). Part of our rationale for evaluating TriMab as a broad-spectrum positive control reagent was that certain combinations of these MAbs have been shown to act synergistically (58, 59, 66, 119), although others have reported a lack of synergism (9, 114). Our results support the utility of TriMab as a broad-spectrum positive control reagent, but it is not clear that the extended range of neutralizing activity was due to synergistic effects. In most cases, the potency of TriMab approximated the potency of the single most effective component in the trivalent mixture. For example, all three components of the TriMab mixture neutralized Env clone 6535.3 as single MAbs. Of these, 2G12 was most potent (ID_{50} of 0.6 μg/ml), whereas TriMab had an ID_{50} of 1.2 μg/ml (equivalent to 0.4 μg 2G12/ml). These results indicate that strong synergy between the three MAbs in TriMab was rare, at least against clade B viruses. We do not rule out the possibility that weak synergy was present in some cases and went unnoticed in our assays.

Additional assessments were made with six MAbs that target epitopes in the V3 loop of gp120 (42–44, 123). Relatively few cases of positive neutralization were detected with these MAbs. SF162.LS and SS1196.1 were notable exceptions in that both were highly sensitive to neutralization by all six MAbs (ID_{50} of <0.04 to 2). Other exceptions included 6535.3, which

TABLE 5. Neutralization phenotypes of pseudoviruses containing cloned Env from acute or early HIV-1 infections as determined with serum from HIV-Igp120-vaccinated subjects

PTID[a]	ID_{50} in TZM-bl assay with W61D-TCLA virus[b]	% RLU reduction in TZM-bl assay[c]							
		6101.10	5768.4	3988.25	7165.18	6535.3*	QH0692.42*	QH0515.1	SC422661.8*
Vaccine recipients									
010	9,582	0	9	0	28	26	40	0	0
008	6,825	0	0	0	26	27	29	0	15
060	3,853	19	25	23	**62**	31	**61**	23	38
042	3,832	**51**	**59**	**52**	**69**	**71**	**72**	**52**	**58**
067	2,767	0	0	0	0	39	0	0	0
055	2,054	0	0	0	14	41	23	0	0
003	1,585	0	0	0	0	42	5	0	0
072	967	0	0	0	8	13	21	0	0
078	796	32	17	33	46	**52**	**53**	33	18
036	771	1	20	0	20	28	10	2	9
064	625	41	**56**	**53**	**57**	**64**	**52**	**51**	38
024	439	26	23	32	45	40	44	29	43
022	270	14	18	0	0	27	23	7	14
034	188	0	0	5	3	0	16	0	7
Control subjects									
026	<10	32	40	41	35	32	30	36	34
040	<10	0	0	0	0	0	0	0	0
001	<10	49	**51**	**53**	**55**	47	**53**	**57**	**53**
058	<10	31	31	46	32	46	39	35	36
013	<10	0	0	0	0	0	0	0	0
044	<10	0	0	0	0	0	0	0	0

[a] PTID, patient identification number. Vaccine recipients received 20 μg gp120 protein in AS02A adjuvant. Control subjects received adjuvant alone.

[b] Serum samples were obtained 2 weeks after the final immunization. Values are the reciprocal serum dilution at which RLU were reduced 50% compared to those in virus control wells.

[c] Samples obtained preimmunization and 2 weeks after the final immunization were screened at a 1:10 dilution in triplicate in TZM-bl cells. Values are percent RLU reduction by postimmune serum compared to the corresponding preimmune serum. Reductions of ≥50% are in boldface. Clones selected as standard reference strains are shown with an asterisk.

was neutralized by five V3-specific MAbs. Also, clones 7165.18 and REJO4541.67 were neutralized by three and two MAbs, respectively. Four additional Env clones (QH0692.42, REJO4541.67, TRJO4551.58, and WITO4160.33) were sensitive to a single V3-specific MAb, while the remaining 10 Env clones were resistant to all V3-specific MAbs tested.

It was important to determine whether the early/acute Env clones were sensitive to neutralization by serum samples from gp120-immunized human subjects. Antibodies elicited by gp120 immunogens often neutralize TCLA strains but exhibit poor neutralizing activity against primary isolates (5, 6, 13, 68). We aimed to determine whether the Env clones selected for study here would yield similar findings. Sera from 14 recipients of a gp120 immunogen and six control subjects in a recent phase I clinical trial were screened at a 1:10 dilution for neutralizing activity against all 19 early/acute Env-pseudotyped viruses (Table 5). Titers of neutralizing Abs against the Env vaccine strain of virus, W61D-TCLA, also were measured. ID_{50} titers against the vaccine strain ranged from 188 to 9,582, including seven samples that had titers of >1,500. Despite this potent neutralization of W61D-TCLA, little or no neutralizing activity against most acute/early Env-pseudotyped viruses was detected. Occasional weak positive neutralizing activity was detected, but this activity was not significant compared to occasional weak false-positive neutralizing activity (51 to 59% reduction in RLU) detected in sera from control subjects. SS1196.1 was an exception in that ≥90% neutralization of this clone was seen with sera from three gp120-immunized subjects, possibly due to the heightened sensitivity of this virus to neutralization by V3-specific antibodies. This Env clone was more sensitive to neutralization by V3-specific MAbs than all other acute/early infection Env clones examined (Table 6) and is highly sensitive to neutralization by V3 loop-specific antibodies generated by a variety of HIV-1 Env immunogens (D. C. Montefiori, unpublished observations). Interestingly, Env clone 6535.3, which was also broadly sensitive to V3-specific MAbs (Table 6), was relatively insensitive to the anti-gp120 serum samples.

Neutralization sensitivities of Env-pseudotyped viruses produced in 293T cells compared to uncloned parental viruses produced in PBMC. Past measurements of HIV-1-specific neutralizing Abs have relied on uncloned virus stocks produced and assayed in human PBMC. Here we used acute/early infection Env clones in pseudoviruses that were produced by transfection in 293T cells and assayed in JC53-BL cells; both of these cell lines are epithelial in origin (40, 45). These new technologies afford significant technical advantages for assay standardization, validation, and high throughput, making them an attractive alternative to PBMC assays. However, the type of cells used to prepare virus stocks has been shown to influence the neutralization phenotype of the virus by either genetic selection (99, 117) or the impact of one or more additional host cell factors (32, 41, 90, 112). Because PBMC are a more natural cell substrate for HIV-1, it was of interest to determine how the neutralization phenotypes of 293T pseudovirions compared to those of PBMC viruses.

The immediate uncloned parental viruses of all 12 molecularly cloned Envs derived originally from PBMC cocultures

TABLE 5—*Continued*

% RLU reduction in TZM-bl assay[c]										
SS1196.1	BG1168.1	PVO.4*	TRO.11*	AC10.0.29*	RHPA4259.7*	THRO4146.18*	REJO4541.67*	TRJO4551.58*	WITO4160.33*	CAAN 5342.A2*
90	0	0	0	0	0	5	21	0	0	0
73	0	5	6	0	5	18	14	6	0	0
91	14	22	47	25	41	**52**	**51**	**56**	41	40
92	**56**	**52**	**51**	47	**59**	**67**	**65**	49	**54**	23
50	0	3	0	0	0	0	0	0	0	0
58	0	0	0	7	5	15	0	0	0	20
40	0	0	0	0	0	4	0	0	0	0
45	0	0	0	6	0	7	0	0	1	0
66	34	26	25	23	40	33	34	36	41	39
44	0	0	0	9	5	4	20	0	0	0
69	39	42	**53**	39	**54**	**62**	**55**	**60**	**52**	20
71	28	37	31	27	30	36	33	37	22	2
50	0	0	0	18	0	1	0	0	0	3
35	0	1	0	2	16	0	5	20	8	0
14	32	28	23	**52**	34	19	37	24	32	32
0	0	0	0	0	0	0	0	0	0	0
55	47	40	**59**	47	49	**54**	**55**	**54**	**56**	40
55	40	32	32	37	27	35	33	**52**	37	11
0	0	0	0	0	0	0	0	0	0	0
0	0	0	1	0	0	0	0	0	0	0

TABLE 6. Sensitivity of Env clones to neutralization by V3-specific MAbs

Env clone[a]	Panel designation[a]	ID_{50} in TZM-bl cells (μg/ml)[b]					
		2182	2442	2191	2219	2456	447D
SF162.LS	None	2	<0.04	<0.04	<0.04	0.04	0.04
6101.10	SVPB1	>25	>25	>25	>25	>25	>25
5768.4	SVPB2	>25	>25	>25	>25	>25	>25
3988.25	SVPB3	>25	>25	>25	>25	>25	>25
7165.18	SVPB4	>25	0.04	0.90	25	>25	>25
6535.3*	SVPB5*	>25	0.04	0.40	1.5	5	0.1
QH0692.42*	SVPB6*	1	>25	>25	>25	>25	>25
QH0515.1	SVPB7	>25	>25	>25	>25	>25	>25
SC422661.8*	SVPB8*	>25	>25	>25	>25	>25	>25
SS1196.1	SVPB9	<0.04	0.04	0.40	0.1	0.1	<0.04
BG1168.1	SVPB10	>25	>25	>25	>25	>25	>25
PVO.4*	SVPB11*	>25	>25	>25	>25	>25	>25
TRO.11*	SVPB12*	>25	>25	>25	>25	>25	>25
AC10.0.29*	SVPB13*	>25	>25	>25	>25	>25	>25
RHPA4259.7*	SVPB14*	>25	>25	25	>25	>25	>25
THRO4156.18*	SVPB15*	>25	>25	>25	>25	>25	>25
REJO4541.67*	SVPB16*	>25	>25	20	1	>25	>25
TRJO4551.58*	SVPB17*	>25	>25	>25	>25	>25	>25
WITO4160.33*	SVPB18*	>25	>25	>25	>25	>25	>25
CAAN5342.A2*	SVPB19*	>25	>25	>25	>25	>25	>25

[a] Clones selected as standard reference strains are marked with an asterisk.
[b] Values are the concentration at which RLU were reduced 50% compared to those in virus control wells.

(Table 1) were assayed with sCD4, MAbs, and a subset of the serum samples used for Table 4. A comparison of ID_{50} values revealed that the 293T Env-pseudotyped viruses were more sensitive to neutralization by a wide spectrum of antibody specificities than were their PBMC parental viruses (Fig. 5). This dichotomy in neutralization sensitivity was best illustrated with serum DUMC-1, which was ~10-fold more potent against 293T pseudoviruses than against PBMC parental viruses. In addition, 293T pseudoviruses were ~22-fold more sensitive to inhibition by sCD4. The greatest discrepancy was seen with 4E10, which was very potent against 293T pseudoviruses (ID_{50} range of 0.1 to 8.5 μg/ml) (Table 4) but had little or no detectable neutralizing activity against PBMC parental viruses (ID_{50} range of 17 to >50 μg/ml) (Fig. 5). Similar results with 4E10 have been reported previously (9). We observed no cases where a 293T pseudovirus was more resistant to neutralization than its PBMC parental virus. These results indicate that Env-pseudotyped viruses produced in 293T cells are more sensitive for detecting neutralizing Abs in JC53-BL cells than are PBMC-grown viruses.

Deciding on an initial panel of standard reference strains. Molecularly cloned gp160s from 19 primary HIV-1 isolates were characterized with the aim of selecting 12 that would be suitable to comprise an initial panel of reference strains for tier 2 and tier 3 assessments of vaccine-elicited neutralizing Abs (64). Emphasis was placed on selecting viruses that are not unusually sensitive or resistant to neutralization by HIV-1-positive serum samples and plasma pools, while also maintaining an ability to detect antibody specificities that resemble those of IgG1b12, 2G12, 2F5, 4E10, and broadly reactive V3-specific MAbs. Priority was given to Env clones obtained from heterosexual individuals recently infected with HIV-1. Genetic and geographic diversities were also considered. The 12 Env clones selected as reference strains are designated with an asterisk in Tables 1 to 6 and Fig. 4, and are shaded in Fig. 3. Three of the original 19 clones examined were excluded on the bases of (i) being too sensitive to neutralization (SS1196.1), (ii) isolated from an individual relatively late in infection (3988.25; 24 weeks from onset of symptoms of acute infection), and (iii) encoding a truncated gp41 cytoplasmic tail (6101.10). An additional four Env clones were excluded because they were the least sensitive to neutralization by the HIV-1-positive serum samples and plasma pools. The decision to exclude these four Env clones was made to improve the ability to detect vaccine-elicited neutralizing Abs. Their exclusion should come at a low risk of overestimating the potential value of a neutralizing Ab response, since the 12 Env clones selected as standard reference strains were relatively insensitive to neutralization by serum samples from subjects who were vaccinated with a prototypic monomeric gp120 immunogen (Table 5).

DISCUSSION

Genetic and antigenic variabilities in HIV-1 are major obstacles to overcome in designing effective vaccines and also pose a challenge for assessing vaccine-elicited immune responses. As a partial solution to the latter challenge, a three-tier approach to assessing vaccine-elicited neutralizing Abs has been recommended (64). In this three-tier approach, serum samples are first tested for their ability to neutralize the vaccine strain(s) of virus and one or more additional strains that are highly sensitive to neutralization (tier 1). Samples that test positive in tier 1 are further tested for cross-neutralizing activity against heterologous primary isolates that are matched in clade to the vaccine strain(s) (tier 2). Samples that test positive in tier 2 are then tested against heterologous strains that belong to other clades (tier 3). This three-tier approach minimizes the number of assays to perform in the case of weak immunogens and allows large data sets to be acquired in cases where stronger immunogenicity is observed.

The main goal of the present study was to devise a panel of full-length molecularly cloned *env* genes from 12 acute/early subtype B infections that may be used as reference reagents for standardized tier 2 and tier 3 assessments of vaccine-elicited neutralizing Abs. Cloned gp160 genes from 19 primary viruses were characterized, with the expectation that not all of these would satisfy the recommended requirements for selection. The 12 clones that were ultimately selected produce high titers of infectious Env-pseudotypes virus when cotransfected with an *env*-minus HIV-1 backbone plasmid in 293T cells, thus allowing reliable measurements of virus neutralization to be made in a rapid, sensitive, and high-throughput format. These clones have been donated to the NIH ARRRP and their sequences deposited in GenBank.

The ideal size and composition of reference panels of HIV-1 strains for standardized assessments of vaccine-elicited neutralizing Abs are uncertain. Based on current evidence, several properties of the viruses are deemed desirable (64), and we aimed to incorporate many of these properties in the clade B panel devised here. One of our highest priorities was to ensure that the viruses were not unusually sensitive or resistant to neutralization by serum and plasma specimens from HIV-1-infected individuals. Only one acute/early infection Env clone proved to be unusually sensitive; this virus, SS1196.1, was highly sensitive to neutralization by V3-specific antibodies.

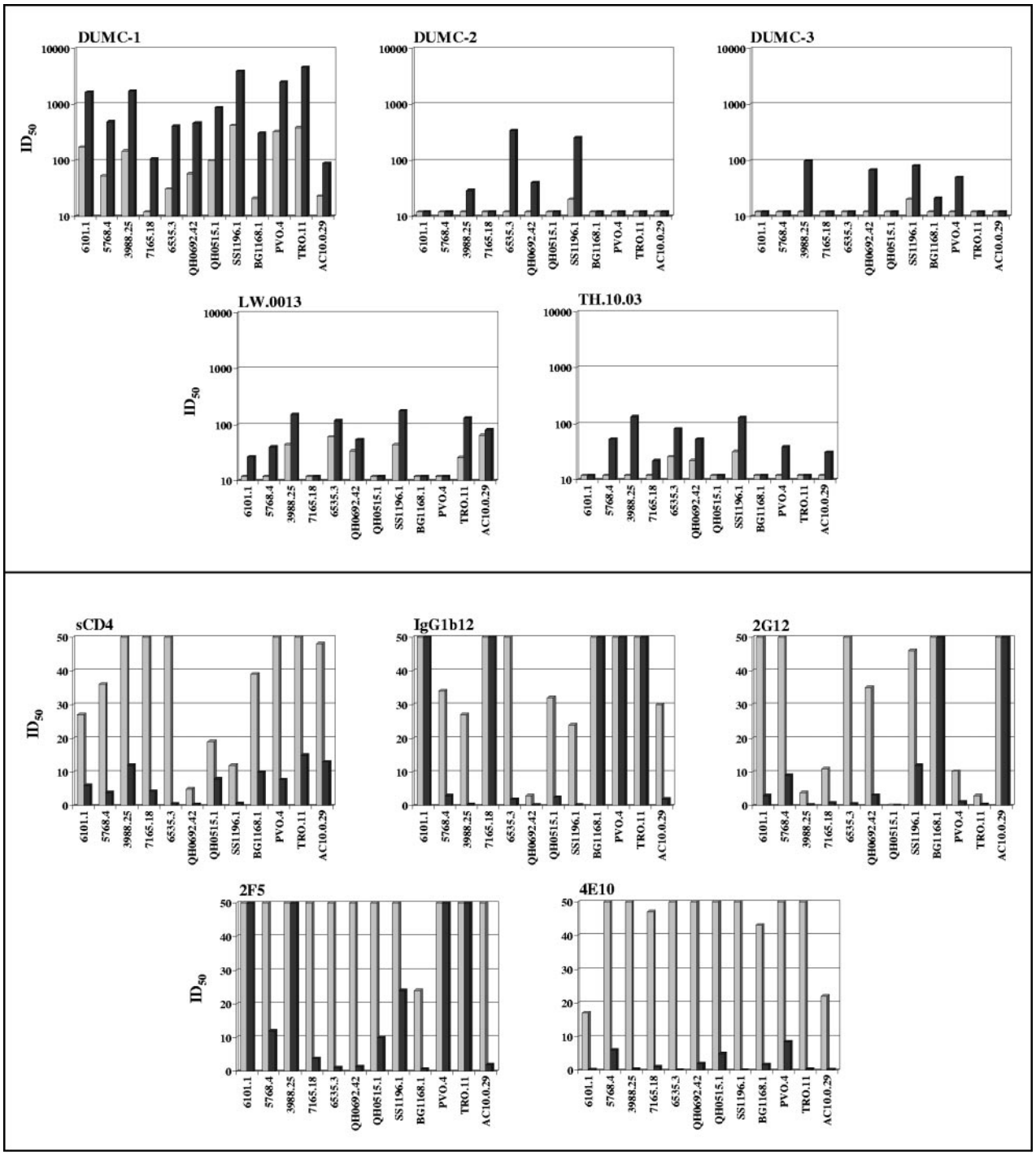

FIG. 5. Neutralization phenotypes of acute/early HIV-1 Env clones compared to the phenotypes of the immediate uncloned parental viruses. Uncloned parental PBMC-grown viruses (gray bars) and the corresponding Env-pseudotyped viruses produced in 293T cells (black bars) were assayed with HIV-1-positive serum samples, sCD4, and human MAbs in JC53-BL cells.

This strain may be more appropriate for use in a tier 1 panel. The remaining viruses selected as reference strains were considerably less sensitive to neutralization and, in this regard, were readily distinguishable from TCLA strains. None of the strains characterized here would be considered unusually resistant to neutralization, since each was sensitive to neutralization by sCD4, multiple HIV-1-positive serum samples, and one or more MAbs.

One unexpected finding was that Env-pseudotyped viruses made in 293T cells were considerably more sensitive to neutralization than their parental uncloned viruses made in PBMC when both viruses were assayed in JC53-BL cells. This may be

viewed as an advantage for assay sensitivity when Env-pseudotyped viruses are used, but it also raises concern that pseudovirions will overestimate the protective value of a neutralizing Ab response. These concerns are diminished when one considers that very little neutralizing activity was detected against Env-pseudotyped viruses when assayed with serum samples from recipients of a prototypic monomeric gp120 immunogen (Table 6). Differences in neutralization sensitivity between viruses made in 293T cells and PBMC might be explained by one or more factors: (i) the relative ease of neutralizing a single clone compared to a viral quasispecies, (ii) differences in the composition of proteins on the surface of the target cells used for infection (93), (iii) glycan composition and number of Env glycoprotein spikes on the virus surface, and/or (iv) factors associated with different adhesion molecules and other cellular proteins that become embedded on the virus surface after assembly and release from various cell types (32, 41, 90, 112). Results of a multicenter comparative assay study indicated that differences in neutralization sensitivity are unrelated to the use of JC53-BL cells compared to PBMC as targets for infection, since similar results were obtained in both cases (Montefiori et al., unpublished data). Experiments with replication-competent, molecularly cloned HIV-1 made in both 293T cells and human PBMC have provided evidence that the cells used for virus synthesis are a major contributor to this effect, where molecular clones made in 293T cells are more sensitive to neutralization (62). In general, we find that 293T Env-pseudotyped viruses are needed in the JC53-BL assay to achieve a level of sensitivity that matches that for the use of PBMC-grown viruses in conventional PBMC assays (72).

The host cell factors that influence virus neutralization remain unknown; however, they did not distort the qualitative detection of neutralizing Abs in our study. In many cases pseudoviruses were as insensitive to neutralization by serum and plasma samples from infected individuals as were their uncloned parental PBMC-grown viruses (Fig. 5). Moreover, we found no evidence that the pseudoviruses were unusually sensitive to neutralization by V3-specific MAbs (Table 5) and sera from gp120-immunized subjects (Table 6). In general, the pseudoviruses appeared to detect the same antibody specificities that were detected with uncloned PBMC viruses but with greater sensitivity. Interestingly, the greatest influence of host cell factors on neutralization susceptibility was observed for the 4E10 epitope. The membrane-proximal location of this epitope might make its access to antibody particularly susceptible to the presence of adjacent cellular proteins on the virus surface.

The reference strains describe here were all obtained soon after sexually acquired infection and thus may represent transmitted viruses that a vaccine will need to protect against. Recent studies suggest that clade A and C viruses from acute and early infection encode compact envelope glycoproteins that are more susceptible to neutralization by donor plasma than virus that is prevalent in chronic HIV-1 infection (20, 25). While this has not been observed for clade B viruses (20, 35), we felt it was important to focus on acute/early isolates when devising reference strains for clade B in the event that transmission generally involves unique genetic and antigenic features, yet to be identified, that could affect the assessment of vaccine-elicited neutralizing Abs.

The panel of reference strains described here should be viewed as an initial panel that may require modification as new information on how to improve its correlative value emerges. Yet, at this time, the selected clade B tier 2 panel provides a valuable tool to facilitate proficiency testing and assay validation in adherence with good laboratory practice guidelines. The initial clade B panel described here will also facilitate the standardized assessment of neutralizing Ab responses generated by the current pipeline of candidate vaccines. A number of scientific issues have been identified (64), and more may be identified over time, that will need to be addressed when deciding whether and how this initial panel should be reconstructed in the future. Information gained from these reference strains and other well-characterized strains (9, 12) will be helpful in making those decisions.

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