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Serotyping of Primary Human Immunodeficiency Virus Type 1 Isolates from Diverse Geographic Locations by Flow Cytometry

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The immunologic relatedness of the various human immunodeficiency virus type 1 (HIV-1) clades was determined with 13 human anti-HIV-1 monoclonal antibodies (MAbs) to six immunogenic regions of the HIV-1 structural proteins. The immunoreactivity of the native, oligomeric viral envelope glycoproteins expressed on the surfaces of human peripheral blood mononuclear cells infected in vitro with primary isolates from clades A through E was determined by flow cytometry. Some epitopes in the immunodominant region of gp41 and the C terminus of gp120 appear to be HIV-1 group specific in that they are expressed on the surfaces of cells in cultures infected with the majority of viruses tested from clades A to E. Epitopes within the V3 region appear to be clade restricted. Surprisingly, one MAb to an epitope in the C terminus of gp120 was entirely clade B specific. Staining with anti-V2 and anti-CD4 binding domain (CD4bd) reagents was infrequently detected. Anti-CD4bd MAbs stained only CD4-negative T cells because the CD4bd of gp120 appeared to be complexed with membrane CD4. When present, the epitopes of V2 and the CD4bd appeared to be expressed on cells infected with various clades. Thus, the results suggest that MAbs to gp41, the C terminus, and the V3 loop of gp120 are most useful in serotyping primary isolates of HIV-1, providing group-specific, clade-restricted, and clade-specific reagents. The use of the immunofluorescent method with the reagents described herein distinguishes infection with clade B from that with all other HIV-1 clades. With additional MAbs, this technique will allow a broadly applicable, reproducible, and practical method for serotyping HIV-1.

In order to map the immunologic relationships among the human immunodeficiency virus type 1 (HIV-1) clades and determine how serotypes parallel or diverge from genotypes, immunologic studies with primary isolates from the various HIV-1 clades and with defined monoclonal antibodies (MAbs) are required. These studies are also needed to characterize the virus within an individual and the virus strains within a defined population, and they are necessary, as well, to provide a foundation for ongoing vaccine development and information about the extent to which critical epitopes are shared by viruses of the various clades of HIV-1.

Preliminary studies of the immunologic relatedness of the various HIV-1 subtypes have been published. Thus, some group-specific epitopes in the HIV-1 envelope have been identified. These include determinants in the immunodominant region of gp41 (6, 53) and the C terminus of gp120 (43). While MAbs to these epitopes have not previously been tested on typed viruses from around the world, these epitopes appear to be present in all or most variants of HIV-1 that have been tested to date.

Antibodies to the CD4 binding domain (CD4bd) of gp120 are also commonly referred to as being group specific since early studies indicated that antibodies to this region cross-react with divergent laboratory isolates (22). Antibodies to the CD4bd have neutralizing activity, but anti-CD4bd MAbs are not capable of neutralizing all HIV-1 strains; thus, of a panel of three human anti-CD4bd MAbs tested against eight type B HIV-1 laboratory isolates, none was able to neutralize all variants (16). Similar results were found by others using both laboratory and primary isolates of HIV-1 (2, 24, 39, 44, 51).

A potentially important group-specific MAb is that reported by Muster et al. (37). This human MAb is directed against the external domain of gp41 in a region that is highly conserved; it is capable of neutralizing many type B isolates of HIV-1 (10) but has not been tested for its ability to react with non-clade B viruses.

To date, no clade-specific antibodies reactive with envelope antigens have been definitively identified. The data of Mascola et al. (32) suggest that polyclonal sera from infected individuals show clade-restricted neutralization patterns in that sera from U.S. and Thai patients preferentially neutralize the homologous clades of U.S. and Thai primary isolates. One human MAb and some rodent anti-V3 (clade B) antibodies (1, 9, 16, 41) appear to react broadly with many clade B viruses, but there are, as yet, no reports as to their reactivity with non-clade B viruses.

Intraclade subdivisions have been distinguished genotypically. Thus, for example, clade F viruses have been divided into two separate categories, with one subclade appearing in Brazil and one occurring in Romania (11, 31, 45). Similarly, intraclade groups have been identified serologically. Thus, Ebenbichler et al. (12) have used a panel of anti-V3 MAbs to classify two distinct V3 loop conformations which correlate with T-cell tropism and macrophage tropism.

Most of the aforementioned studies of immunologic relationships among HIV-1 viruses have been performed with laboratory isolates or with untyped primary isolates. In order to define these relationships more precisely, we have chosen to use human MAbs to analyze the expression of epitopes on peripheral blood mononuclear cells (PBMCs) infected in vitro with primary viruses from five different clades. These human

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MAbs were induced by native viral proteins during the course of natural infection and were selected on the basis of their reactivity with proteins or peptides of laboratory strains of clade B. Using these methods and materials, we have been able to define group-specific, clade-restricted, and clade-specific epitopes of HIV-1.

MATERIALS AND METHODS

Viruses used. Virus preparations were provided by three sources: (i) the Military Medical Consortium for Applied Retroviral Research and the Henry M. Jackson Foundation for Military Medicine (MMCARR/HMJF), (ii) the World Health Organization (WHO), and (iii) the National Institute of Allergy and Infectious Diseases (NIAID). The methods of virus isolation and preparation have been described previously (7, 47). Briefly, virus was isolated by coculture of phytohemagglutinin (PHA)-stimulated PBMCs from infected and uninfected individuals. For expansion to high-titered stocks, culture supernatants from the initial isolates were used to infect PHA-stimulated PBMCs from healthy, uninfected individuals. Cultures were monitored for p24 production, and supernatant was collected and stored in 1-ml aliquots in liquid nitrogen. None of the primary isolates was passaged through neoplastic cell lines, and the viruses used throughout were uncloned; each virus preparation therefore contains a population of viruses. A PBMC-adapted stock of HIV_{LAI} was obtained from A. Prince. This virus was prepared and tested in the same manner as that described above for primary isolates.

The assignment of each virus to a clade was based on multiple genetic analyses (54). Amino acid sequences were described by Myers et al. (38) or were provided by MMCARR/HMJF, WHO, or NIAID. Viruses received from WHO and NIAID are referenced according to the nomenclature now used by Myers et al. (38). Thus, for example, isolate A2RW021 denotes that this isolate belongs to clade A (A), was collected in 1992 (indicated as 2), was derived from a patient in Rwanda (RW), and was assigned specimen number 021. Isolates provided by MMCARR/HMJF do not conform to this identification scheme. Country codes include RW, Rwanda; US, United States; BZ or BR, Brazil; TH, Thailand; HA, Haiti; SM, Somalia; SG, Senegal; and UG, Uganda. The designations for virus phenotypes were provided by each of the contributing agencies. In cases in which analyses of syncytium-inducing and non-syncytium-inducing phenotypes had not been performed by the virus provider, such analyses were performed as part of this study by means of the MT-2 assay of Koot et al. (30).

The following virus isolates were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, from the WHO Network for HIV Isolation and Characterization: A2RW021W, A2RW023W, A2RW026W, B2TH014W, B2TH026W, C2BR025W, D2UG001W, D2UG021W, D2UG024W, E2TH001W, E2TH003W, and E2TH005W.

The following isolates were obtained through the Division of AIDS, NIAID: B2US657D and B2US727D from the Multicenter AIDS Cohort Study, from Roger Detels and John Phair, respectively; B2HA593D, B2HA594D, and B2HA599D from Neal Halsey, Johns Hopkins University; and B2US714D from Kenrad Nelson, Johns Hopkins University.

Preparation of cultured, infected cells for flow cytometry. PBMCs from uninfected donors were prepared by Ficoll-Hypaque density centrifugation from Leukopaks purchased from the New York Blood Center. The Leukopaks consisted of whole blood, collected in CPDA-1 (citrate, phosphate, dextrose, and adenine solution), from which plasma had been removed. These fresh PBMCs, at a concentration of 2×10^6 cells per ml, were stimulated for 3 days with 5 μg of PHA per ml (Difco Laboratories, Detroit, Mich.) in medium containing 20% fetal calf serum, 2% penicillin-streptomycin (Bio-Whittaker, Walkersville, Md.), and 1% L-glutamine (Bio-Whittaker). Cultures were carried out in tissue culture flasks incubated in 7% CO_2 at 37°C. After 3 days, the cells were readjusted to a concentration of 2×10^6 cells per ml in the medium described above, except that the medium lacked PHA and was supplemented with 20 U of recombinant interleukin-2 per ml (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Cells (5 ml) and virus (1 ml) containing a minimum of 20 50% tissue culture infectious doses per ml were incubated together for 30 min at 37°C, and then 4 ml of interleukin-2-supplemented medium was added. The cultures were maintained with feeding every 2 days, spun, decanted, and resuspended in 10 ml of medium (as delineated above) until the number of CD4-positive cells decreased by at least 10% as measured by flow cytometry (see below), usually 4 to 8 days. The same procedure was used for primary isolates and for PBMC-adapted HIV_{LAI}. For uninfected negative control cultures, virus-free medium was added in place of virus; all other steps in infected and uninfected cultures were identical.

Antibodies used for staining. The mouse anti-CD4 MAb Leu-3a (Becton Dickinson, San Jose, Calif.) was used to identify CD4-positive cells. The human anti-HIV-1 MAbs used for analysis and the epitopes for which they are specific are shown in Table 1. Human MAbs were used to determine if antibodies representative of those produced by the human immune response could distinguish between viruses from different geographic locations and if they could define serotypes. The human MAbs used were chosen from a panel of over 60. They were selected on the basis of the diversity of epitopes with which they react, and those used were MAbs whose epitopes had been well characterized (Table 1 and references therein). Maximal staining was achieved at MAb concentrations of ≥0.5 μg/ml. Staining levels remained constant when MAb concentrations of up to 80 μg/ml were used. Three human MAbs to the C terminus of gp120 which have not previously been described were used in these studies. Each of these was produced by heterohybridomas derived from the PBMCs of HIV-infected individuals as previously described (17, 19), and they were selected by screening supernatants on recombinant gp120 (rgp120) from HIV_{LAI} (Repligen, Cambridge, Mass.). The anti-C terminus MAbs were mapped by their ability to react with peptides from the C terminus of rgp120 (data not shown).

TABLE 1. Human MAbs used

MAb	Epitope	Reference(s)
71-31	p24	17
50-69	gp41 (amino acids 579–613)	17, 56
858-D	gp120 (C terminus: VVQREKR)	This study
670-D	gp120 (C terminus: PTKAKRR)	This study
989-D	gp120 (C terminus: VVQREKR)	This study
257-D	gp120 (V3 loop: RKIHI)	19, 20
268-D	gp120 (V3 loop: HIGPGR)	19, 20
447-52-D	gp120 (V3 loop: GPGR)	16, 20
694/98-D	gp120 (V3 loop: GRAF)	16, 20
697-D	gp120 (V2 loop)	18
559/64-D	gp120 (CD4 binding domain)	28
654-D	gp120 (CD4 binding domain)	33
729-D	gp120 (CD4 binding domain)	33

Unpurified MAbs in culture supernatants were used to avoid any aggregation which might occur upon purification and which might lead to an increase in nonspecific background staining levels. A pool of sera from five HIV-positive subjects was also used to stain cells; this serum pool was used at a dilution of 1:1,000.

Staining of cells and flow cytometric analysis. For single-color staining, 10^6 washed cells (in 100 μl) which had been cultured as described above were incubated with 100 μl of a given human MAb for 30 min at 4°C. Following incubation, the cells were washed once by centrifugation in phosphate-buffered saline containing 0.1% sodium azide (PBS-azide). To the cell pellet was added 50 μl of a 1:8 dilution of phycoerythrin (PE)-conjugated goat Fc-specific anti-human immunoglobulin G (IgG) (Caltag, South San Francisco, Calif.). After 30 min of incubation at 4°C, the cells were again washed once, resuspended in PBS-azide, and analyzed by flow cytometry with a FACScan (Becton Dickinson). For single-color analyses, the cells were gated on live lymphocytes using forward and 90° scatter; the negative peak was defined with infected cells stained with goat anti-human IgG-PE in the absence of any human anti-HIV MAb.

For two-color analyses, the cultured cells were incubated with 100 μl of the designated human anti-HIV MAb and 15 μl of fluorescein isothiocyanate-labeled mouse anti-CD4 MAb (Becton Dickinson) for 30 min at 4°C and then were washed once with PBS-azide. A 1:8 dilution of the goat anti-human IgG-PE (50 μl) was then added, incubation was continued for 30 min at 4°C, and after they were washed once, the cells were resuspended and analyzed. For dual-color analyses, the cells were gated on scatter as described above. The cells were regated on CD4-positive cells to determine the mean channel fluorescence of CD4-positive cells stained with anti-HIV MAbs.

For three-color analyses, in addition to human anti-HIV MAbs and fluorescein isothiocyanate-labeled anti-CD4, Per CP anti-CD3 (Becton Dickinson) was added to the cultured cells in the initial incubation. The cells were gated by scatter and regated on HIV-positive, CD4-negative cells to determine the percentage of the latter which were CD3 positive.

Flow cytometry was carried out with a Becton Dickinson FACScan. The instrument sensitivity and reproducibility were verified with reference particles (CaliBRITE Beads; Becton Dickinson). Compensation was set with fluorochrome-labeled reference particles and optimized on normal control cell mixtures. Data on one to three experiments performed with each virus are reported. When more than one experiment was performed (for 8 of the 28 viruses studied), data are shown as the mean; repeat analyses were performed on newly infected cells. Each analysis was performed on cells infected with viruses as they were received, and thus viruses of several different clades were usually represented in each experiment that was conducted.

Statistical methods. The MAbs were grouped into clusters by the techniques of cluster analysis (26). Similarities between MAbs were based on the patterns of response (positive and negative) of cells infected with HIV isolates to the MAbs, and these similarities were quantified by the average linkage algorithm. This algorithm treats the distance between two clusters as the average distance between all pairs of items for which one member of a pair belongs to each cluster. Initially, all MAbs are in separate clusters. The most similar MAbs are then grouped, and these initial groups are subsequently merged according to their

FIG. 1. Percentages of cells in cultures infected with primary isolates (or HIV_{LAI}) which stain positively with human MAbs. Values in color are in excess of the mean value + 2 standard deviations for cells in infected cultures stained with anti-p24 as a negative control (3.39% + 2.8% = 6.19%). C-TERM, C terminus; NSI, non-syncytium inducing; SI, syncytium inducing; N.D., not done.

similarities. Eventually, all subgroups are fused into a single cluster. SAS statistical software was used for the analysis.

RESULTS

Staining characteristics of HIV_{LAI}-infected cells. To ascertain the ability of a panel of MAbs to stain antigens on the surfaces of HIV-1-infected cells, PBMCs infected with a PBMC-adapted variant of HIV_{LAI} were stained with each of 13 human MAbs. An anti-p24 MAb (MAb 71-31) was used as a negative control. With this latter MAb, only 2.9% of cultured cells were stained over the gate set with infected cells which were treated with goat anti-human IgG-PE in the absence of any human anti-HIV MAb. Anti-V3 MAbs 257-D and 268-D had previously been shown to be unreactive with HIV_{LAI} (19, 20); these two MAbs served as additional negative controls in this experiment and were found to stain 2.6 and 2.5% of infected cells, respectively. In 15 experiments, staining levels with the anti-p24 MAb used as a negative control reached a mean + 2 standard deviations of 6.2%, which was hereinafter used as the cutoff between negative and positive values. The additional 12 anti-HIV MAbs tested, which were specific for antigenic determinants of gp41 and gp120, had previously been shown to react with HIV_{LAI} (16, 17, 19, 20, 28), and each stained >6.2% of cells (Fig. 1). Three anti-CD4bd MAbs known to react with rgp120 HIV_{LAI} stained between 7.0 and 8.0% of cells. An anti-V2 MAb (697-D) stained 9.2% of cells, and an anti-gp41 MAb (50-69) stained 27.1% of cells. Two MAbs to the V3 loop, 447-52-D and 694/98-D, known to react with and neutralize HIV_{LAI}, stained 19.1 and 58.4% of cells in HIV_{LAI}-infected cultures, respectively. The differential staining of these two anti-V3 MAbs is consistent with their respective binding affinities for $rgp120_{LAI}$ and their ability to neutralize HIV_{LAI} (19, 20). Other MAbs which were also reactive with the cells in the HIV_{LAI}-infected culture were the MAbs to the C terminus of gp120 (MAbs 670-D, 858-D, and 989-D), which stained 62.8, 57.6, and 48.3% of the cells, respectively. Thus, among the reactive MAbs, the hierarchy of surface staining by antibody specificity for various epitopes was (in order of ascending percentages of cells stained) anti-CD4bd < anti-V2 < anti-gp41 < anti-V3 ~ anti-C terminus. Uninfected cells did not stain above background levels with any anti-HIV MAb (Fig. 1) but did stain with anti-CD4 (data not shown).

The hierarchy of staining ability was similar to that derived when the data were analyzed for mean channel fluorescence (MCF) rather than for percent positive cells. Thus, the average MCF value for the three MAbs that did not stain the cells was 37.3. The MCF value for three anti-CD4bd MAbs was 39, that for one anti-V2 MAb was 49, that for one anti-gp41 MAb was 70, that for two anti-V3 MAbs was 86, and that for three anti-C terminus MAbs was 116.

Because the anti-CD4bd MAbs showed only a small percentage of positively stained cells (7.0 to 8.0%) and the MCF value of CD4-positive cells stained with anti-CD4bd MAbs was essentially unchanged above the negative control values, the nature of the cells binding the anti-CD4bd and other anti-HIV MAbs was investigated. Figure 2a shows that neither CD4-negative nor CD4-positive cells stained with the anti-p24 MAb 71-31. Figure 2b and c show that both CD4-negative and CD4-positive cells stained intensely with the anti-V3 and anti-C terminus MAbs, respectively. However, Fig. 2d, e, and f show that three anti-CD4bd MAbs stained only CD4-negative cells. These data suggest that on CD4-positive cells, the CD4bd of

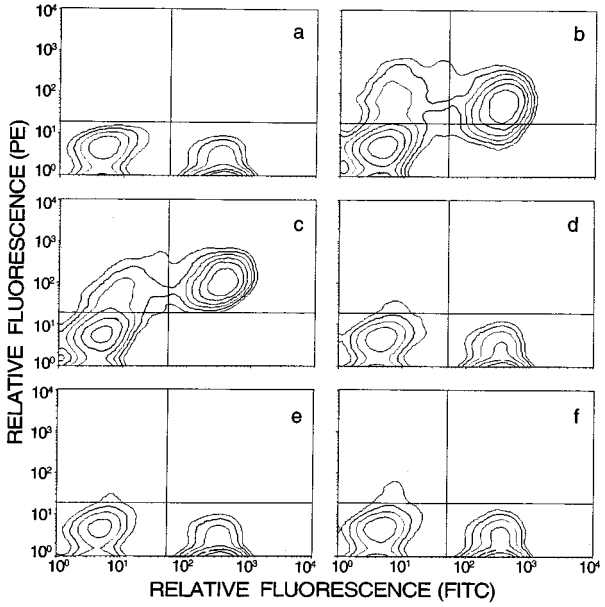

FIG. 2. Relationship of CD4 and HIV antigen expression determined by flow cytometric analysis of PHA-stimulated PBMCs which had been infected 4 days earlier with a PBMC-adapted strain of HIV_{LAI}. Contour maps of cells stained with fluorescein isothiocyanate (FITC)-labeled anti-CD4 mouse MAb and with human anti-HIV MAbs and PE-conjugated goat Fc-specific anti-human IgG are shown. The intensity of the fluorescein isothiocyanate staining (anti-CD4) is shown on the *x* axis, and the intensity of the PE staining (anti-HIV) is shown on the *y* axis. Human anti-p24 MAb 71-31 (a), anti-V3 MAb 694/98-D (b), anti-C terminus (gp120) MAb 670-D (c), anti-CD4bd MAb 559/64-D (d), anti-CD4bd MAb 654-D (e), and anti-CD4bd MAb 729-D (f) were used.

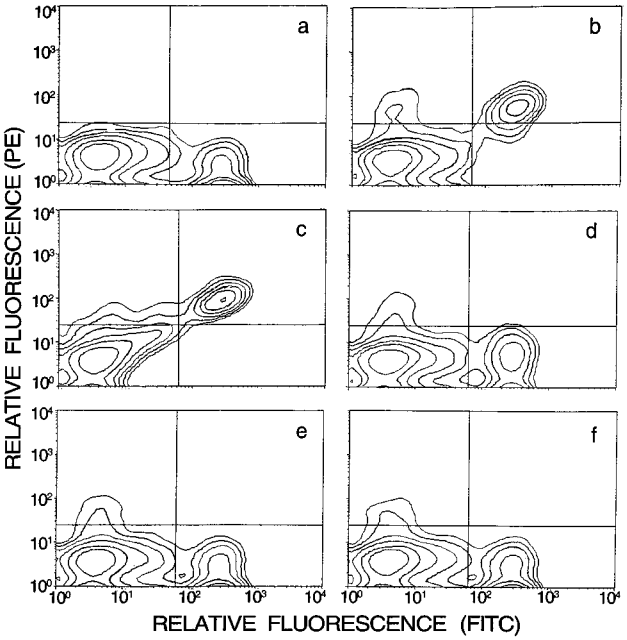

FIG. 3. Relationship of CD4 and HIV antigen expression determined by flow cytometric analysis of PHA-stimulated PBMCs which had been infected 4 days earlier with primary isolate B2HA593D. Contour maps are shown, and the details of the staining are identical to those delineated in the legend to Fig. 2. FITC, fluorescein isothiocyanate.

gp120 may be complexed with CD4, thus blocking the CD4bd. However, since HIV-1 infection down-regulates CD4 (15, 25, 48), the CD4bd should be available on infected cells which no longer express CD4. This appears to be supported by the data shown.

Staining characteristics of cultures infected with primary isolates. In order to ascertain whether cells in cultures infected with primary isolates were stained in a pattern similar to that observed with the cells infected with the HIV_{LAI} clade B laboratory strain, normal PBMCs were infected with one of several clade B viruses. The results, summarized in Fig. 1, show that cells in cultures infected with primary isolates of clade B can, indeed, be stained with anti-gp41, anti-C terminus, anti-V3, anti-CD4bd, and anti-V2 MAbs. The details of the staining profiles with these reagents are presented in Fig. 1 and are described below. Figure 3 shows the contour maps of cells in cultures infected with a clade B virus, B2HA593D, which were stained with anti-CD4 and various anti-HIV MAbs. The similarity of the staining patterns of cells in cultures infected with this primary isolate to those of cells infected with the PBMC-adapted strain of HIV_{LAI} is evident from a comparison of Fig. 2 and 3. Thus, essentially no cells were stained with anti-p24 (Fig. 2a and 3a), cells were stained strongly with anti-V3 and anti-C terminus MAbs among both CD4-negative and CD4-positive populations (Fig. 2b and c and 3b and c), and CD4-negative cells were essentially the only cells which were stained with the anti-CD4bd MAbs (Fig. 2d to f and 3d to f). Three-color analysis demonstrated that 88% of the infected CD4-negative cells were CD3-positive T cells (data not shown). Moreover, time course experiments showed that the CD4bd on CD4-negative cells was the last epitope to be detected, and this occurred at a time when the number of cells expressing CD4 had begun to decline dramatically (data not shown). These data support the hypothesis that the CD4bd is blocked by membrane CD4 but is available for binding on cells whose CD4 has been down-regulated.

The staining intensity of the primary isolates with the different categories of MAbs was analyzed next. For those CD4-positive cells from infected cultures that were stained positively with anti-HIV MAbs, staining was weakest with anti-CD4bd MAbs, intermediate with anti-gp41 and anti-V2 MAbs, and strongest with anti-C terminus and anti-V3 MAbs. Examples of the intensity of the staining of the cells infected with clade B primary isolates can be discerned from the contour maps in Fig. 3 and from the histograms in Fig. 4. It should be noted that the intensities of staining achieved with polyclonal antibodies from a pool of sera from HIV-infected individuals (Fig. 4g), with an anti-C terminus MAb (Fig. 4e), and with an anti-V3 MAb (Fig. 4f) were similar (MCFs were 138.1, 128, and 133.5, respectively).

Group-specific antigens recognized on the surfaces of cells in cultures infected with HIV-1 primary isolates from diverse locales. After the ability of various human anti-HIV MAbs to stain PBMCs in cultures infected with laboratory and primary isolates of clade B was established, the ability of these MAbs to stain PBMCs in cultures infected with various primary isolates from diverse geographic locations was tested. Virus isolates from clades A to E were used to infect PHA-stimulated human PBMCs. Three MAbs were found to stain cells in cultures infected with most of the viruses tested (Fig. 1). Thus, MAb 50-69 specific for the immunodominant domain of gp41 (56) stained cells in cultures infected with 21 of 27 (78%) of the primary viruses tested. Similarly, MAbs 670-D and 858-D specific for the C terminus of gp120 stained cells in cultures infected with 100% (n = 19) and 81% (n = 27) of the viruses tested, respectively. In each case, cells in cultures infected with viruses from each of the five clades were among those which

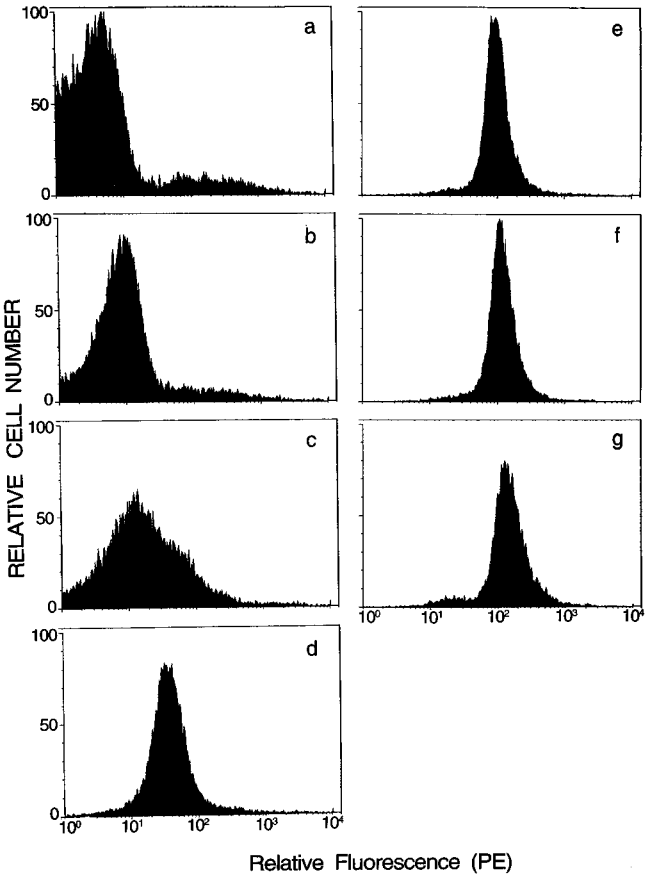

FIG. 4. Fluorescence histograms of CD4-positive PHA-stimulated PBMCs which had been infected 4 days earlier with primary isolate US1. The intensities of the staining with human anti-p24 MAb 71-31 (a), anti-CD4bd MAb 559/64-D (b), anti-gp41 MAb 50-69 (c), anti-V2 MAb 697-D (d), anti-C terminus (gp120) MAb 670-D (e), and anti-V3 MAb 447-52D (f) are shown. In panel g, the cells were stained with a 1:1,000 dilution of sera pooled from five HIV-positive subjects.

stained positively. These data confirm the findings of others that antigens from the immunodominant domain of gp41 and from the C terminus of gp120 appear to be ubiquitous among HIV-1 isolates and are, therefore, group-specific antigens (43, 53). The frequency with which cells from infected cultures stained positively with anti-C terminus MAbs ranged from 8 to 82% (Fig. 1). This is not dissimilar to previous reports that 50 to 100% of cells infected with HIV in vitro express antigens that can be detected by flow cytometry (5, 23, 29, 55).

Clade-restricted antigens on the surfaces of cells in cultures infected with HIV-1 primary isolates. Four anti-V3 MAbs were tested for their ability to recognize antigens on the surfaces of cells in infected cultures. These MAbs had been derived from the cells of North American HIV-positive patients and had been shown to react with the V3 loop from clade B viruses. These MAbs reacted primarily with cells in cultures infected with viruses from clade B (Fig. 1). Eleven of the 14 primary clade B viruses tested contain the GPGR motif at the crown of the loop. Staining this group of 11 primary isolates with the four anti-V3 MAbs resulted in positive values in 38 of 44 (86%) analyses. Of the three clade B viruses which had divergent sequences at the crown of the V3 loop, two were from Thailand (B2TH026W and TH237) and one was from Haiti (B2HA599D). The two Thai viruses differed from the majority of viruses tested here by having PLGPGQ and HLGPGK (instead of HIGPGR) at the tip of the loop. The aberrant Haitian virus had a 3-amino-acid insertion near the crown of the loop and GGGR at the tip (Table 2). These viruses are hereinafter categorized as clade B′ on the basis of their aberrant binding to the four anti-V3 MAbs. Only 1 of 12 (8%) analyses of these clade B′ viruses resulted in cells stained positively with anti-V3 MAbs. The data suggest that most of the type B viruses from around the world, including those from the United States, Brazil, Haiti, and Thailand, can be identified with anti-V3 (clade B) human MAbs.

The anti-V3 MAb 447-52D, which is specific for the GPGR sequence at the crown of the V3 loop, reacted with cells infected with all of the viruses bearing this sequence (Table 2); this MAb has previously been shown to be broadly neutralizing for clade B primary and laboratory isolates (9, 16). MAb 694/98-D has GRAX as its core epitope; it stained cells infected with 10 of 11 viruses having this sequence in the V3 loop. This MAb, more than any of the other anti-V3 MAbs, is affected by conformation-dependent factors, as it was selected on rgp120 rather than on a V3 peptide (16, 20). MAb 257-D, whose core epitope was previously defined as KRIHI (19), stained cells in cultures infected with 9 of 11 clade B primary viruses; those not stained or which stained poorly (viruses BZ167 and B2US714D) were infected with viruses with a substitution in at least one residue within this epitope (Table 2). MAb 268-D, whose core epitope was previously defined as HIGPGR (19), stained cells in cultures infected with 8 of 11 clade B primary viruses; cells that were not stained were infected with viruses with a substitution for the histidine in this epitope (Table 2). These data show the remarkably consistent sequence-dependent staining by anti-V3 MAbs.

Two of the anti-V3 MAbs showed reactivity with cells infected with one of three clade A viruses. The staining of the cells cultured with this clade A virus (A2RW023) was strong and unequivocal in three replicate experiments.

One anti-C terminus MAb, 989-D, was entirely clade B specific but was reactive with only 7 of 11 (64%) clade B primary viruses and with two of three (67%) clade B′ viruses.

Expression of V2 on cells in cultures infected with primary isolates. Only one human MAb to the V2 region, MAb 697-D, has been identified to date. The epitope of this MAb appears to span the region from positions 161 to 194 of gp120 (the position numbering is based on HIV_{HXB2R} numbering [38]), and it is primarily conformation dependent, with weak reactivity for a linear region between amino acids 161 and 180 (18). This anti-V2 MAb reacted sporadically with viruses from clades B, B′, C, and D (Fig. 1). Although no consistent sequence which differentiated the V2 sequences of those viruses that generated cells that were stained with 697-D from those viruses that did not (data not shown) could be identified, a particularly frequent variation was noted in the V2 region from positions 164 to 172 (SIRGKVQKE), a region which was previously noted to be involved in the epitope of MAb 697-D (18). In addition, a correlation was observed (Fig. 1) for those cultures which stained with the anti-CD4bd MAbs. This was confirmed by cluster analysis (see below).

Expression of CD4bd on cells infected with primary isolates. As noted above, the staining of cells in infected cultures with anti-CD4bd MAbs was sporadic because of the blockade of the CD4bd by CD4; only infected CD4-negative cells stained with anti-CD4bd MAbs. The staining, when it did occur, was found on cells infected with viruses from clades A, B, C, and D. The data are consistent with previously published results that the CD4bd may be broadly distributed among HIV-1 viruses (27, 35); however, immunofluorescence does not appear to be an

TABLE 2. Correlation of staining with anti-V3 MAbs and V3 sequences of isolates used to infect PBMCs

Virus designation	V3 sequence	Cells (%) staining with MAb:				Sequence in virus corresponding to core epitope of MAb
		257-D	268-D	447-52D	694/98D	
US4	KSIHI---GPGRAF	41	42	41	28	KSIHI HIGPGR GPGR GRAF
BZ167	RRIRI---GPGRTF			43	19	GPGR GRTF
B2THO14W	KSIHL---GPGRAW	17	16	18	13	KSIHL HLGPGR GPGR GRAW
TH130	KRIHI---GPGRAF	43	44	44	23	KRIHI HIGPGR GPGR GRAF
US1	KSIHI---GPGRAI	78	78	79	77	KSIHI HIGPGR GPGR GRAI
US2	KSIHI---GPGRAF	73	74	64	43	KSIHI HIGPGR GPGR GRAF
B2US714D	RSIHM---GPGRAF		15	16	17	HMGPGR GPGR GRAF
B2HA594D	KRISI---GPGRAS	28		35	23	KRISI GPGR GRAS
B2HA593D	KRISI---GPGRAF	21		24	28	KRISI GPGR GRAF
B2US657D	KGIHI---GPGRAF	39	40	40	28	KGIHI HIGPGR GPGR GRAF
B2US727D	KSVHI---GPGRAF	12	23	14		KSVHI HIGPGR GPGR GRAF
LAI	KSIRI-QRGPGRAF			19	58	GPGR GRAF
B2TH026W	KSIPL---GPGQAW					
B2HA599D	RSVHSGHIGGGRTL					
TH237	KSIHL---GPGKAW	28				KSIHL

optimal technique for assessing its distribution among the various clades.

Cluster analysis of the data. The results of the cluster analysis were utilized to construct a phenogram (not shown) which describes the history of the clustering process and suggests the existence of immunologic relationships as a result of the reactivities of the cells from infected cultures with the MAbs specific for various epitopes. This analysis suggests the following clusters.

(i) The anti-gp41 MAb and anti-C terminus MAb 858-D cluster together. (Analysis of anti-C terminus MAb 670-D could not be performed because of an incomplete data set with this MAb; inspection of Fig. 1, however, suggests that MAb 670-D belongs in this cluster.) On the basis of the ubiquity of these epitopes among the various clades, this cluster appears to reflect an immunologic association that identifies the relevant epitopes as being group specific.

(ii) The V3 MAbs tend to cluster together among themselves and with 989-D, an anti-C terminus MAb. On the basis of the restricted reactivity of this cluster, it appears to reflect the immunologic association which identifies the relevant epitopes as clade related. The two most closely related pairs of MAbs in this cluster are MAbs 447-52D and 694/98-D and MAbs 257-D and 268-D. These relationships confirm the overlap noted in the mapping of the core epitopes of these MAbs (Table 2).

(iii) The anti-CD4bd MAbs cluster together and with the anti-V2 MAb. This finding supports the notion of a relationship between the V2 loop and the CD4bd on both immunochemical and functional bases. This relationship has been described previously (14, 34, 46).

DISCUSSION

The serotyping of infectious organisms is the classical technique used to categorize of organisms within a given genus or species. This approach allows the differentiation of strains of bacteria and viruses on the basis of antigenic structures which are recognized with specific antisera or MAbs and provides information on shared immunologic and structural characteristics. Thus, for example, poliovirus is divided into serotypes primarily on the basis of epitopes located on the three structural proteins making up the viral surface (13, 42, 52). Similar immunologic, or serotypic, analyses have been used to categorize most virus families, subfamilies, and genera (13). More recently, with the development of techniques in molecular biology, serotypic analyses of many bacteria and viruses have been augmented with genotypic classification wherein organisms are categorized by restriction endonuclease mapping or by sequence analysis at the nucleotide level and/or amino acid level.

Like most viruses, HIV-1 and HIV-2 were first distinguished by serologic methods. Thus, Barin et al. (3) and Clavel et al. (8) described sera from West African subjects which reacted more strongly with simian immunodeficiency virus than with HIV-1. Virus isolation and sequence analysis later revealed that this West African virus, HIV-2, was distinct from HIV-1 and that these two viruses represented two different species within the lentivirus genus (21).

In contrast to the typing of most viruses, subclassification of HIV-1 was initially based on nucleotide sequence analysis rather than on serotyping. More than six HIV-1 subtypes, or clades, have now been definitively identified, and they are approximately equidistant from one another on a coding sequence tree (38). Viruses of each clade tend to cluster in geographic areas.

In order to determine how these viruses were immunologically related, whether serotypes exist, and if so, whether serotypes parallel the genotypic groupings, we studied the reactivity of antigens expressed on the surfaces of PBMCs cultured with primary isolates from each of five different genotypes (clades A to E). Flow cytometry was used, as opposed to studying the antigens detected in detergent-treated lysates of virions by immunochemical techniques, to examine the antigenic structure of oligomeric viral glycoproteins in their native configuration on the surfaces of cells rather than partially denatured monomeric glycoproteins captured onto a plastic substrate. Moreover, in order to analyze these antigens in the context of the human anti-HIV immune response, MAbs derived from the cells of HIV-1-infected humans were used.

To standardize the method, MAbs were first tested for their ability to stain cells infected with a PBMC-adapted laboratory isolate, HIV_{LAI}; all of the MAbs to the HIV-1 glycoproteins which had previously been shown to react immunochemically with recombinant proteins and peptides of HIV_{LAI} were able to stain cells infected with this virus. Interestingly, the patterns of the staining of cells infected with HIV_{LAI} and with clade B primary isolates were strikingly similar (Fig. 1 to 3).

The method used, acute infection of stimulated PBMCs with free virus, gave excellent levels of infected cells expressing viral

antigens. The mean percentage of cells stained with the most sensitive and broadly reactive MAbs, the two group-specific anti-C terminus MAbs, was 36%. While the range of percent positive cells in cultures stained with these two MAbs extended from 8 to 82%, the level of infection was consistent with those in studies in the literature showing that 50 to 100% of transformed cells infected in vitro are infected (5, 23, 29, 55) and that ~30% of acutely infected PBMCs in vitro are infected (23). These figures are strikingly higher than those for the level of infection achieved by cocultivation of activated cells from normal and infected donors, which results in a much lower level of infection that peaks much later (40, 57).

Analysis of the immunologic nature of HIV-1 envelope glycoproteins in their native configuration on the surfaces of infected cells revealed various categories of epitopes.

Group-specific epitopes appear on cells infected with the majority of HIV-1 isolates, regardless of clade. Included in this category are an immunodominant epitope of gp41 located between amino acid residues 579 and 613 and recognized by MAb 50-69 (17, 56) and two distinct antigens at the C terminus of gp120 recognized by MAbs 670-D and 858-D. Previous studies have suggested that the immunodominant antigen on gp41 is partially obscured by its association with gp120 (48–50); this appears to be confirmed here on the basis of the lower level of fluorescence obtained with this MAb compared with that for MAbs that stained other envelope epitopes.

Clade-restricted epitopes appear on cells in cultures infected with only some or one of the HIV-1 clades. Surprisingly, there appears to be a clade B-restricted epitope in the C terminus of gp120 which is recognized by MAb 989-D. Clade-specific epitopes in this region have not, heretofore, been described, although Blomberg et al. (4) showed that African sera reacted preferentially with peptides from the C terminus of gp120 of two Zairian viruses. While the MAb that recognizes the clade-specific C terminus epitope maps to the same peptide to which the group-specific anti-C terminus MAb 858-D maps (VVQREKR), the two MAbs clearly differ in the patterns of viruses they recognize (Fig. 1). Since the specificity of MAbs to linear determinants is determined in large measure by conformation (16), this unusual epitope may be primarily conformational, thus providing an explanation for the absence of information about this determinant from sequence analysis. Studies to delineate the different epitopes of several anti-C terminus MAbs are continuing.

The other category of clade-restricted epitopes was recognized by anti-V3 MAbs. Two anti-V3 MAbs recognized cells in cultures infected with viruses of clade B and with viruses of clade A or clade B′ (those with aberrant V3 loops compared with those of most clade B viruses). Two other anti-V3 MAbs reacted with cells in cultures infected with only clade B viruses, suggesting the existence of a category of clade-specific anti-V3 epitopes. Since V3 is one of the principal neutralizing determinants in primary isolates (9), the data herein support the concept that the V3 loop epitope is important in defining a functionally relevant (neutralizing) serotype. The various anti-V3 MAbs may be useful not only in distinguishing between clades but in making distinctions within clades. Thus, while only three clade B′ viruses were available for testing, these were distinguished from the clade B viruses by the anti-V3 MAbs. Such intratypic subgroups have previously been reported for clade F, as determined by genotypic methods (31). The immunologic data presented herein suggest that at least one intratypic subgroup within clade B may now be discerned immunologically.

It is noteworthy that these various anti-V3 MAbs react broadly with clade B virus-infected cultures despite the hypervariability of the V3 loop (Table 2). The reactivity of the four anti-V3 MAbs in 86% of the analyses with 11 clade B primary viruses suggests the existence of an immunologic similarity between diverse isolates even in this variable region. Human MAbs which were stimulated by natural infection as opposed to MAbs induced by peptides may be particularly adept at recognizing the conserved shape of the clade B V3 loop. Indeed, all of these human anti-V3 MAbs have been shown to bind more avidly to rgp120 than to V3 peptides (16, 20), demonstrating a required conformational aspect of the antigen in order for binding to be maximal.

A third category of epitopes can be detected only sporadically on cells in cultures infected with isolates from the various clades. The best example of this type of epitope is that recognized by the anti-V2 MAb 697-D on cells in cultures infected with a minority of viruses from clades B, B′, C, and D. The V2 epitope was expressed on both CD4-positive and CD4-negative cells (data not shown) in 30% of the analyses performed with cultures infected with primary isolates. Similarly, anti-CD4bd MAbs stained cells infected by a minority of viruses from clades A to D, staining cells in 23% of the analyses performed. When cells stained with anti-CD4bd MAbs were analyzed, it was found that only cells that failed to stain for CD4 with Leu-3a stained with the anti-CD4bd MAbs. Interestingly, a relationship between the presence of the V2 and CD4bd epitopes appears to exist, and this relationship can be seen by an inspection of the data (Fig. 1) and by cluster analysis. Evidence for functional and immunochemical relationships between V1/V2 and the C4 region and CD4bd of gp120 has previously been described (14, 36, 46).

The V2 and CD4bd epitopes may be variant specific, i.e., present on cells in cultures infected with only a minority of viruses which, however, are not restricted by membership in any particular clade. Alternatively, one or both of these epitopes may be more widespread but poorly detected on the surfaces of infected cells because of the blockade of the epitopes by gp120 (15). At a minimum, one can state that shared antigens of the CD4bd are found among most of the clades (35), but the degree to which they are shared by individual viruses cannot be ascertained by staining infected PBMCs. Previous studies of virus neutralization (2, 16, 24, 39, 44, 51) showed that only a portion of viruses studied can be neutralized by anti-CD4bd MAbs, suggesting that, on this functional criterion, some antigens of this large and complex epitope may fall into the variant-specific rather than the group-specific category.

Quantitative rather than qualitative analysis of the data highlights additional issues. Thus, each of the MAbs tested stained cells in infected cultures to various degrees. For example, the anti-C terminus MAb 670-D stained all infected cultures tested, but the level of staining varied from 19 to 82% (Fig. 1). The level of staining with the anti-gp41 MAb 50-69 ranged from 2.2 to 27.1%. This range in the staining by a single MAb of cells infected with different viruses and the differences noted in staining by different MAbs of cells infected with a single virus may be due to several nonexclusive factors: the nonclonal nature of the infecting viruses, differences in the affinities of given MAbs for any given virus glycoprotein, the various states of glycoprotein processing and glycosylation on the surfaces of infected cells, and the degree to which different primary viruses shed monomeric gp120 which may bind to the surfaces of uninfected cells.

Definition of group-specific and clade-specific antigens and a panel of MAbs that would define each will be useful in epidemiologic studies and in some clinical settings, particularly in countries where infection is caused by multiple clades. With

these reagents, definition of the dominant clades causing infection in a defined geographic region or in an individual could be performed relatively simply by fluorescence microscopy, and the need for isolation, sequencing, and/or production of the relatively large amounts of virus needed for immunochemical analysis could be avoided (35). The MAbs to group-specific antigens would serve as positive controls; the clade-specific MAbs would distinguish between clades. Given the current assumption that reagents for active and/or passive immunization will need to match the locally dominant virus (or the virus in an infected pregnant mother), readily available information about the serotypes of HIV-1 in populations and in individuals is essential.

To date, the vast majority of anti-HIV-1 human MAbs have been derived from PBMCs from North American and European HIV-infected individuals and selected on proteins and peptides of clade B viruses. These have yielded reagents that are useful in categorizing group-specific epitopes, clade-restricted epitopes (which react with clade A-, B-, and B′-infected cells), and clade-specific epitopes (which react exclusively with clade B-infected cells). Studies are now under way to develop MAbs derived from the cells of individuals infected with non-B clades in order to complete a panel of MAbs for serotyping which will contain clade-specific reagents reactive with each of the individual clades of HIV-1. These reagents and the methodology described in this paper provide a practical approach for the serotyping and categorizing of strains of HIV-1 without the sequencing or growing of large quantities of the virus.

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REFERENCES

1. **Akerblom, L., J. Hinkula, P.-A. Broliden, B. Makitalo, T. Fridberger, J. Rosen, M. Villacres-Eriksson, B. Morein, and B. Wahren.** 1990. Neutralizing cross-reactive and non-neutralizing monclonal antibodies to HIV-1 gp120. AIDS **4:**953–960.
2. **Arendrup, M., A. Sonnerborg, B. Svennerholm, L. Akerblom, C. Nielsen, H. Clausen, S. Olofsson, J. O. Nielsen, and J.-E. S. Hansen.** 1993. Neutralizing antibody response during human immunodeficiency virus type 1 infection: type and group specificity and viral escape. J. Gen. Virol. **74:**855–863.
3. **Barin, F., S. M'Boup, F. Denis, P. Kanki, J. S. Allan, T. H. Lee, et al.** 1985. Serological evidence for virus related to simian T-lymphotropic retrovirus III in residents of West Africa. Lancet **ii:**1387–1389.
4. **Blomberg, J., A. Lawoko, R. Pipkorn, S. Moyo, B. E. Malmvall, J. Shao, R. Dash, and S. Tswana.** 1993. A survey of synthetic HIV-1 peptides with natural and chimeric sequences for differential reactivity with Zimbabwean, Tanzanian and Swedish HIV-1-positive sera. AIDS **7:**759–767.
5. **Bou-Habib, D. C., G. Roderiquez, T. Oravecz, P. W. Berman, P. Lusso, and M. A. Norcross.** 1994. Cryptic nature of envelope V3 region epitopes protects primary monocytotropic human immunodeficiency virus type 1 from antibody neutralization. J. Virol. **68:**6006–6013.
6. **Bugge, T. H., B. O. Lindhardt, L. L. Hansen, P. Kusk, E. Hulgaard, K. Holmback, P. J. Klasse, J. Zeuthen, and K. Ulrich.** 1990. Analysis of a highly immunodominant epitope in the human immunodeficiency virus type 1 transmembrane glycoprotein, gp41, defined by a human monoclonal antibody. J. Virol. **64:**4123–4129.
7. **Burke, D. S., A. K. Fowler, R. R. Redfield, S. Dilworth, and C. N. Oster.** 1990. Isolation of HIV-1 from the blood of seropositive adults: patient stage of illness and sample inoculum size are major determinants of a positive culture. J. Acquired Immune Defic. Syndr. **3:**1159–1167.
8. **Clavel, F., D. Guetard, F. Brun-Vezinet, S. Chamaret, M. A. Rey, O. Santos-Ferreira, et al.** 1986. Isolation of a new human retrovirus from West African patients with AIDS. Science **233:**343–346.
9. **Conley, A. J., M. K. Gorny, J. A. Kessler II, L. J. Boots, M. Ossorio-Castro, S. Koenig, D. W. Lineberger, E. A. Emini, C. Williams, and S. Zolla-Pazner.** 1994. Neutralization of primary human immunodeficiency virus type 1 isolates by the broadly reactive anti-V3 monoclonal antibody, 447-52D. J. Virol. **68:**6994–7000.
10. **Conley, A. J., J. A. Kessler, L. J. Boots, J.-S. Tung, B. A. Arnold, P. M. Keller, A. R. Shaw, and E. A. Emini.** 1994. Neutralization of divergent human immunodeficiency virus type 1 variants and primary isolates by IAM-41-2F5, an anti-gp41 human monoclonal antibody. Proc. Natl. Acad. Sci. USA **91:** 3348–3352.
11. **Dumitrescu, D., M. L. Kalish, S. C. Klicks, C. I. Bandia, and J. A. Levy.** 1994. Characterization of human immunodeficiency virus type-1 isolates from children in Romania: identification of a new envelope subtype. J. Infect. Dis. **169:**281–288.
12. **Ebenbichler, C., P. Westervelt, A. Carrillo, T. Henkel, D. Johnson, and L. Ratner.** 1993. Structure-function relationships of the HIV-1 envelope V3 loop tropism determinant: evidence for two distinct conformations. AIDS **7:**639–646.
13. **Fields, B. W., and D. M. Knipe.** 1990. Virology. Raven Press, New York.
14. **Freed, E. O., and M. A. Martin.** 1994. Evidence for a functional interaction between the V1/V2 and C4 domains of human immunodeficiency virus type 1 envelope glycoprotein gp 120. J. Virol. **68:**2503–2512.
15. **Geleziumas, R., S. Bour, and M. Wainberg.** 1994. Cell surface down-modulation of CD4 after infection by HIV-1. FASEB J. **8:**593–600.
16. **Gorny, M. K., A. J. Conley, S. Karwowska, A. Buchbinder, J.-Y. Xu, E. A. Emini, S. Koenig, and S. Zolla-Pazner.** 1992. Neutralization of diverse human immunodeficiency virus type 1 variants by an anti-V3 human monoclonal antibody. J. Virol. **66:**7538–7542.
17. **Gorny, M. K., V. Gianakakos, S. Sharpe, and S. Zolla-Pazner.** 1989. Generation of human monoclonal antibodies to HIV. Proc. Natl. Acad. Sci. USA **86:**1624–1628.
18. **Gorny, M. K., J. P. Moore, A. J. Conley, S. Karwowska, J. Sodroski, C. Williams, S. Burda, L. J. Boots, and S. Zolla-Pazner.** 1994. Human anti-V2 monoclonal antibody that neutralizes primary but not laboratory isolates of human immunodeficiency virus type 1. J. Virol. **68:**8312–8320.
19. **Gorny, M. K., J.-Y. Xu, V. Gianakakos, S. Karwowska, C. Williams, H. W. Sheppard, C. V. Hanson, and S. Zolla-Pazner.** 1991. Production of site-selected neutralizing human monoclonal antibodies against the third variable domain of the HIV-1 envelope glycoprotein. Proc. Natl. Acad. Sci. USA **88:**3238–3242.
20. **Gorny, M. K., J.-Y. Xu, S. Karwowska, A. Buchbinder, and S. Zolla-Pazner.** 1993. Repertoire of neutralizing human monoclonal antibodies specific for the V3 domain of HIV-1 gp120. J. Immunol. **150:**635–643.
21. **Guyader, M., M. Emerman, P. Sonigo, F. Clavel, L. Montagnier, and M. Alizon.** 1987. Genome organization and transactivation of the human immunodeficiency virus type 2. Nature (London) **326:**662–669.
22. **Haigwood, N. L., C. B. Barker, K. W. Higgens, P. V. Skiles, G. K. Moore, K. A. Mann, D. R. Lee, J. W. Eichberg, and K. S. Steimer.** 1990. Evidence for neutralizing antibodies directed against conformational epitopes of HIV-1 gp120, p. 313–320. *In* F. Brown, R. M. Chanock, H. Ginsberg, and R. A. Luner (ed.), Vaccines 90: modern approaches to new vaccines including prevention of AIDS. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Heynen, C. A., and T. J. Holzer.** 1992. Evaluation of a flow cytometric model for monitoring HIV antigen expression in vitro. J. Immunol. Methods **152:** 25–33.
24. **Ho, D. D., J. A. McKeating, X. L. Li, T. Moudgil, E. S. Daar, N.-C. Sun, and J. E. Robinson.** 1991. Conformational epitope on gp120 important in CD4 binding and human immunodeficiency virus type 1 neutralization identified by a human monoclonal antibody. J. Virol. **65:**489–493.
25. **Jabbar, M. A., and D. P. Nayak.** 1990. Intracellular interaction of human immunodeficiency virus type 1 (ARV-2) envelope glycoprotein gp160 with CD4 blocks the movement and maturation of CD4 to the plasma membrane. J. Virol. **64:**6297–6304.
26. **Johnson, R. A., and D. W. Wichern.** 1988. Applied multivariate statistical analysis. Prentice-Hall, Inc., Englewood Cliffs, N.J.
27. **Kang, C.-Y., P. Nara, S. Chamat, V. Caralli, T. Ryskamp, N. Haigwood, R. Newman, and H. Kohler.** 1991. Evidence for non-V3 specific neutralizing antibodies that interfere with gp120/CD4 binding in human immunodeficiency virus 1-infected humans. Proc. Natl. Acad. Sci. USA **88:**6171–6175.
28. **Karwowska, S., M. K. Gorny, A. Buchbinder, V. Gianakakos, C. Williams, T. Fuerst, and S. Zolla-Pazner.** 1992. Production of human monoclonal antibodies specific for conformational and linear non-V3 epitopes of gp120. AIDS Res. Hum. Retroviruses **8:**1099–1106.
29. **Kim, P. K., S. He, and J. L. Ho.** 1994. Rapid culture and quantitation of human immunodeficiency virus type 1 from patient cells without the use of mitogen-stimulated donor cells. Clin. Diagn. Lab. Immunol. **1:**660–666.
30. **Koot, M., A. H. V. Vos, R. P. M. Keet, R. E. DeGoede, M. W. Dercksen, F. G.**

Terpstra, et al. 1992. HIV biological phenotype in long-term infected individuals evaluated with an MT-2 cocultivation assay. J. Acquired Immune Defic. Syndr. **6:**49–54.

31. **Louwagie, J., E. L. Delwart, J. I. Mullins, F. E. McCutchan, G. Eddy, and D. S. Burke.** 1994. Genetic analysis of HIV-1 isolates from Brazil reveals the presence of two distinct genetic subtypes. AIDS Res. Hum. Retroviruses **10:**561–567.
32. **Mascola, J. R., J. Louwagie, F. E. McCutchan, C. L. Fischer, P. A. Hegerich, K. F. Wagner, A. K. Fowler, J. G. McNeil, and D. S. Burke.** 1994. Two antigenically distinct subtypes of HIV-1: viral genotypes predicts neutralization immunotype. J. Infect. Dis. **169:**48–54.
33. **McKeating, J. A., J. Bennett, S. Zolla-Pazner, M. Schutten, S. Ashelford, A. L. Brown, and P. Balfe.** 1993. Resistance of a human serum-selected human immunodeficiency virus type 1 escape mutant to neutralization by CD4 binding site monoclonal antibodies is conferred by a single amino acid change in gp120. J. Virol. **67:**5216–5225.
34. **Moore, J. P., and D. D. Ho.** 1993. Antibodies to discontinuous or conformationally sensitive epitopes on the gp120 glycoprotein of human immunodeficiency virus type 1 are highly prevalent in sera of infected humans. J. Virol. **67:**863–875.
35. **Moore, J. P., F. E. McCutchan, S.-W. Poon, J. Mascola, J. Liu, Y. Cao, and D. D. Ho.** 1994. Exploration of antigenic variation in gp120 from clades A through F of human immunodeficiency virus type 1 by using monoclonal antibodies. J. Virol. **68:**8350–8364.
36. **Moore, J. P., Q. J. Sattentau, H. Yoshiyama, M. Thali, M. Charles, N. Sullivan, S.-W. Poon, M. S. Fung, F. Traincard, M. Pinkus, G. Robey, J. E. Robinson, D. D. Ho, and J. Sodroski.** 1993. Probing the structure of the V2 domain of human immunodeficiency virus type 1 surface glycoprotein gp120 with a panel of eight monoclonal antibodies: human immune response to the V1 and V2 domains. J. Virol. **67:**6136–6151.
37. **Muster, T., F. Steindl, M. Purtscher, A. Trkola, A. Klima, G. Himmler, F. Ruker, and H. Katinger.** 1993. A conserved neutralizing epitope on gp41 of human immunodeficiency virus type 1. J. Virol. **67:**6642–6647.
38. **Myers, G., B. Korber, S. Wain-Hobson, K.-T. Jeang, L. E. Henderson, and G. N. Pavlakis.** 1994. Human retroviruses and AIDS. Theoretical Biology and Biophysics, Los Alamos National Laboratories, Los Alamos, N.Mex.
39. **Nakamura, G. R., R. Byrn, D. M. Wilkes, J. A. Fox, M. R. Hobbs, R. Hastings, H. C. Wessling, M. A. Norcross, B. M. Fendly, and P. W. Berman.** 1993. Strain specificity and binding affinity requirements of neutralizing monoclonal antibodies to the C4 domain of gp120 from human immunodeficiency virus type 1. J. Virol. **67:**6179–6191.
40. **Ohlsson-Wilhelm, B. M., J. M. Cory, H. A. Rersten, M. E. Eyster, F. Rapp, and A. Landy.** 1990. Circulating human immunodeficiency virus (HIV) p24 antigen-positive lymphocytes: a flow cytometric measure of HIV infection. J. Infect. Dis. **162:**1018–1024.
41. **Ohno, T., M. Terada, Y. Yoneda, K. W. Shea, R. F. Chambers, D. M. Stroka, M. Nakamura, and D. W. Kufe.** 1991. A broadly neutralizing monoclonal antibody that recognizes the V3 region of human immunodeficiency virus type 1 glycoprotein gp120. Proc. Natl. Acad. Sci. USA **88:**10726–10729.
42. **Osterhaus, A. D. M. E., A. L. von Wezel, B. von Steenis, G. A. Drost, and A. G. Hazendonk.** 1981. Monoclonal antibodies to poliovirus. Production of specific monoclonal antibodies to the Sabin vaccine strains. Intervirology **16:**218–224.
43. **Palker, T. J., T. J. Matthews, M. E. Clark, G. J. Cianciolo, R. R. Randall, A. J. Langlois, G. C. White, B. Safai, R. Snyderman, D. P. Bolognesi, and B. F. Haynes.** 1987. A conserved region at the COOH terminus of human immunodeficiency virus gp120 envelope protein contains an immunodominant epitope. Proc. Natl. Acad. Sci. USA **84:**2479–2483.
44. **Posner, M. R., L. A. Cavacini, C. L. Emes, J. Power, and R. Byrn.** 1993. Neutralization of HIV-1 by F105, a human monoclonal antibody to the CD4 binding site of gp120. J. Acquired Immune Defic. Syndr. **6:**7–14.
45. **Potts, K. E., M. L. Kalish, T. Lott, G. Orloff, C. C. Luo, M. A. Bernard, C. B. Alves, R. Badaro, J. Suleiman, O. Ferreira, G. Schochetman, W. D. Johnson, C. Y. Ou, Jr., J. L. Ho, and The Brazilian Collaborative AIDS Research Group.** 1993. Genetic heterogeneity of the V3 region of the HIV-1 envelope glycoprotein in Brazil. AIDS **7:**1191–1197.
46. **Roben, P., J. P. Moore, M. Thali, J. Sodroski, and C. F. Barbas III.** 1994. Recognition properties of a panel of human recombinant Fab fragments to the CD4 binding site of gp120 that show differing abilities to neutralize human immunodeficiency virus type 1. J. Virol. **68:**4821–4828.
47. **Rubsamen-Waigmann, H., H. von Briesen, A. Bjorndal, H. Holmes, B. Korber, and other participants in the WHO Network for HIV Isolation and Characterization.** 1994. Standard conditions of virus isolation reveal biological variability of HIV-1 in different regions of the world. AIDS Res. Hum. Retroviruses **10:**1401–1408.
48. **Sattentau, Q. J., and J. P. Moore.** 1991. Conformational changes induced in the human immunodeficiency virus envelope glycoprotein by soluble CD4 binding. J. Exp. Med. **174:**407–416.
49. **Sattentau, Q. J., S. Zolla-Pazner, and P. Poignard.** 1995. Epitope exposure on functional, oligomeric gp41 molecules. Virology **206:**713–717.
50. **Spear, G., D. M. Takefman, B. L. Sullivan, A. L. Landay, and S. Zolla-Pazner.** 1992. Complement activation by human monoclonal antibodies to human immunodeficiency virus. J. Virol. **67:**53–59.
51. **Tilley, S. A., W. J. Honnen, S. Warrier, M. E. Racho, T.-C. Chou, M. Girard, E. Muchmore, M. Hilgartner, D. D. Ho, M. S. C. Fung, and A. Pinter.** 1992. Potent neutralization of HIV-1 by human and chimpanzee monoclonal antibodies directed against three distinct epitope clusters of gp120, p. 211–216. *In* M. Girard and L. Valette (ed.), Retroviruses of human AIDS and related animal diseases. Sixieme Colloque des Cent Guardes, Pasteur-Merieux, Paris.
52. **von Wezel, A. L., and A. G. Hazendonk.** 1979. Intratypic serodifferentiation of polio myelitis virus strains by strain specific antisera. Intervirology **11:**2–8.
53. **Wang, J. J. G., S. Steel, R. Wisniewolski, and C. Y. Wang.** 1986. Detection of antibodies to human T-lymphotropic virus type III by using a synthetic peptide of 21 amino acid residues corresponding to a highly antigenic segment of gp41 envelope protein. Proc. Natl. Acad. Sci. USA **83:**1659–1663.
54. **WHO Network for HIV Isolation and Characterization.** 1994. HIV-1 variation in WHO-sponsored vaccine evaluation sites: genetic screening, sequence analysis and preliminary biological characterization of selected viral strains. AIDS Res. Hum. Retroviruses **10:**1327–1353.
55. **Wrin, R., T. P. Loh, J. C. Vennari, H. Schuitemaker, and J. F. Nunberg.** 1995. Adaptation to persistent growth in the H9 cell line renders a primary isolate of human immunodeficiency virus type 1 sensitive to neutralization by vaccine sera. J. Virol. **69:**39–48.
56. **Xu, J.-Y., M. K. Gorny, T. Palker, S. Karwowska, and S. Zolla-Pazner.** 1991. Epitope mapping of two immunodominant domains of gp41, the transmembrane protein of human immunodeficiency virus type 1, using ten human monoclonal antibodies. J. Virol. **65:**4832–4838.
57. **Zolla-Pazner, S.** Unpublished data.

ISOLATE	SUBTYPE	PHENO-TYPE	71-31 (p24)	50-69 (gp41)	670-D (C-TERM)	858-D (C-TERM)	989-D (C-TERM)	257-D (V3)	268-D (V3)	447-52-D (V3)	694/98-D (V3)	559/64-D (CD4bd)	654-D (CD4bd)	729-D (CD4bd)	697-D (V2)
A2RW021W	A	NSI	2.1	6.4	43.3	26.6	1.5	2	1.6	2	1.6	2.3	2.3	4.1	2.3
A2RW026W	A	NSI	3.2	9.5	N.D.	44	3.2	3.6	2.3	3.8	5.8	3.3	3.2	8.6	2.3
A2RW023W	A	N.D.	2.3	12.7	46.8	33.3	2.7	44.1	1.7	3.3	15.2	2.2	2.9	4.7	2.5
US4	B	NSI	3.5	14.9	54.6	45.3	26.6	41	41.5	41.1	28	4	4.6	6	3.5
BZ167	B	SI	5.2	16.9	50.4	31.4	12.3	4.4	3.9	43.3	19.4	12.2	10.8	14.2	14.6
B2TH014W	B	NSI	2.8	6.7	N.D.	14.5	4.2	16.5	15.8	17.5	12.6	3	2.9	3.9	2.1
TH130	B	SI	4.4	12.9	N.D.	35	21.3	43	44	43.5	23	2.8	3.4	6.8	3.1
US1	B	SI	6.9	27	74.9	65	52.4	78.4	77.8	79.2	76.9	11.5	8.3	14	58.7
US2	B	SI	4.7	9.9	82.1	29.6	15.8	73	73.9	63.9	43	4.3	3.5	6.2	5
B2US714D	B	NSI	3.2	12	40.9	30.1	22.5	4	14.9	16.2	17.3	3.5	3.4	5.9	6.7
B2HA594D	B	SI	4.3	6.4	36.9	15.3	4.4	28.2	3.5	34.6	23.1	3.8	3.2	N.D.	2.8
B2HA593D	B	NSI	4.9	11.4	32	26.9	20.5	21.2	5.8	24.4	27.9	10.3	7	7.5	3.2
B2US657D	B	N.D.	2.5	8.2	42.4	5	2.7	39.3	39.7	39.8	27.9	1.5	1.9	3.1	2.6
B2US727D	B	N.D.	2.2	6.4	37.3	9.6	3.4	12.3	23	13.8	5.2	1.2	1.3	2.3	1.3
HIV(LAI)	B	SI	2.9	27.1	62.8	57.6	48.3	2.6	2.5	19.1	58.4	7	7.2	8	9.2
B2TH026W	B'	NSI	2.3	5	N.D.	4.2	3.5	2.7	1.9	1.7	2.4	2.3	2	3.1	2.6
B2HA599D	B'	SI	2.9	7.4	45	24.9	12.9	2.7	2.5	5.1	1.8	2.3	1.6	3.6	7.4
TH237	B'	NSI	3.6	15.6	40.1	31.5	15.3	27.6	3.3	4.2	5.2	3.9	3.3	3.3	2.3
SM145	C	NSI	3.8	9.9	N.D.	31	2.4	2.9	2	3.3	2.9	3.2	3.5	4.7	3.5
SG364	C	NSI	7	20.1	71.7	54	5	5.9	5	5.7	4.7	7.8	7.2	11.1	7.6
C2BR025W	C	N.D.	2.7	4.8	25.3	8.4	2.3	2.8	2.2	2.4	2.7	2.2	2.8	2.7	2
D2UG021W	D	SI	2.3	6.8	N.D.	13.5	1.6	2	2	2	2.3	7	6.8	8.6	6.9
D2UG001W	D	SI	2.7	14.8	N.D.	20	2.6	2.1	2	1.6	1.9	5.7	5.3	6.4	1.2
D2UG024W	D	SI	4.7	2.8	35	13.8	3	4.7	4.4	1.9	2.4	5.5	4.1	6.8	4.6
D3UG059W	D	N.D.	3.1	4.8	31.7	5.6	3.2	3	3.1	3.1	3.1	3.7	4.7	4.5	7
E2TH003W	E	NSI	1.2	2.2	18.7	2	1.3	1.2	1.2	1.3	1.1	1.1	1	1.4	1.4
E2TH001W	E	NSI	5.5	16.3	79.6	18.3	4.1	4.6	4.2	4.7	3.8	4.3	4.3	5.8	5
E2TH005W	E	NSI	1.9	4.8	20.2	2.3	1.1	1.6	1.2	1	1.1	1.2	1.1	2.2	1.5
NONE	_	_	2.7	2.2	2.6	2	2	2.6	2.4	2.4	2.3	2.2	2.1	3.1	2.3