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At least five distinct genetic subtypes(genotypes) of HIV 1 have been identified by DNA sequencing. Current vaccine candidates are based on virus strains from North America & Europe that represent only one subtype. The extent to which distinct genotypes of HIV-1 correspond to antigenically distinguishable serotype is largely unknown and may be critically important to vaccine design. Cross-neutralization studies were done with viruses & plasma from 2 different genotypes. Based on neutralization susceptibility, 10 primary HIV-1 isolates from Thailand & the U.S. were classified into one of two antigenic subtypes that correlated with viral genotype. The existence of serotypes of HIV-1 suggests that a broadly effective vaccine may have to include strains from multiple subtypes. Neutralization of these primary HIV-1 isolate differed substantially from results with laboratory strains. Future neutralization studies using primary isolates and multiple genotypes may be important for assessment of HIV-1 antigenic diversity.

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## Two Antigenically Distinct Subtypes of Human Immunodeficiency Virus Type 1: Viral Genotype Predicts Neutralization Serotype

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At least five distinct genetic subtypes (genotypes) of human immunodeficiency virus type 1 (HIV-1) have been identified by DNA sequencing. Current vaccine candidates are based on virus strains from North America and Europe that represent only one subtype. The extent to which distinct genotypes of HIV-1 correspond to antigenically distinguishable serotypes is largely unknown and may be critically important to vaccine design. Cross-neutralization studies were done with viruses and plasma from two different genotypes. Based on neutralization susceptibility, 10 primary HIV-1 isolates from Thailand and the United States were classified into one of two antigenic subtypes that correlated with viral genotype. The existence of serotypes of HIV-1 suggests that a broadly effective vaccine may have to include strains from multiple subtypes. Neutralization of these primary HIV-1 isolates differed substantially from results with laboratory strains. Future neutralization studies using primary isolates and multiple genotypes may be important for assessment of HIV-1 antigenic diversity.

An important unanswered question in human immunodeficiency virus type 1 (HIV-1) vaccine development is whether the considerable genetic variability of HIV-1 has immunologic relevance. Analysis of the DNA sequence of internationally collected HIV-1 isolates reveals at least five genetically distinct subtypes that are widely dispersed and intermixed geographically [1-4]. If these genotypes correspond to antigenically distinct serotypes, then to be effective, any candidate vaccine will have to induce an immune response to a wide range of antigenically diverse isolates. Most vaccine development efforts have focused on several prototype (laboratory) HIV-1 strains obtained from individuals in North America or Europe [5-8]. By DNA sequence analysis, these strains cluster in a single genotype [1]. Thus, currently

available vaccine candidates represent only one of the multiple genetic subtypes of HIV-1 that exist worldwide.

To gain a better understanding of the relationship between genetic diversity (genotypes) and antigenic diversity (serotypes), we did cross-neutralization experiments with plasma and viruses representing two distinct HIV-1 genotypes. In several previous reports, serum or plasma from HIV-1-infected individuals from various geographic locales demonstrated strong binding and neutralizing activity to one particular laboratory strain, HIV-1<sub>MN</sub>. It was therefore postulated that viruses prevalent in most parts of the world were antigenically similar to North American or European HIV-1 strains [9-14]. We investigated whether primary wild-type HIV-1 isolates from Thailand and the United States (representing two distinct genotypes) could be distinguished by neutralizing antibody assay and whether viral genotype predicts an antigenically distinct neutralization serotype. Furthermore, we compared neutralizing activity measured against laboratory-adapted HIV-1 strains (strains cultured in neoplastic T cell lines) and plasma neutralizing activity against primary HIV-1 isolates grown in human peripheral blood mononuclear cells (PBMC).

Two distinct genetic subtypes of HIV-1 are prevalent in Thailand [15-17]. Of 16 Thai isolates initially evaluated in this laboratory, a combination of *gag* and *env* DNA sequencing and polymerase chain reaction (PCR) typing revealed that one subtype was similar to viruses prevalent in North America and Europe while the other was a genetically distinct subtype [17]. For neutralization studies, 6 Thai viruses representing both genotypes were selected. Four additional isolates were obtained from infected individuals in the United States.

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Whole blood samples were obtained from patients after approval from the appropriate institutional human subjects review board and informed consent were obtained.

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The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Departments of the Army, Navy, or Defense.

The following DNA sequence data has been submitted to GenBank, Los Alamos National Laboratory, Los Alamos, New Mexico: CM237, L14570; CM238, L14571; CM240, L14572; US1, L14573; US2, L14574; US3, L14575; US4, L14576.

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## Materials and Methods

**Virus and plasma collection.** Heparinized whole blood samples from HIV-1-seropositive persons were transported from Thailand at room temperature and processed within 48 h. Plasma was recovered by centrifugation at 300 g for 15 min and stored in 1-mL aliquots at  $-70^{\circ}\text{C}$ . Virus was isolated by cocultivation of Ficoll-separated PBMC with phytohemagglutinin (PHA)-stimulated donor PBMC as previously described [18]. Four additional virus isolates and plasma were obtained from HIV-1-seropositive persons in the United States. For expansion to high-titered virus stocks, culture supernatant from the initial virus isolation was used to infect  $2 \times 10^7$  fresh PHA-stimulated donor PBMC. Cultures were followed for production of p24 antigen, and supernatant collected on day 7 or 10 was stored in 1-mL aliquots in the vapor phase of liquid nitrogen. None of these primary isolates was passaged through neoplastic T cell lines. To prepare laboratory HIV-1 strains (HIV-1<sub>MN</sub>, HIV-1<sub>IIB</sub> and HIV-1<sub>RF</sub>), supernatant from chronically infected H9 cells was used to infect human PBMC as described above.

**Genetic characterization of virus isolates.** Each of the 10 primary HIV-1 isolates was genetically characterized by full gp160 DNA sequence and phylogenetic tree analysis. DNA from PBMC cocultures was PCR amplified with primers flanking the *env* gene of HIV-1 as previously described [17], except that PCR primers (given 5'-3') were JL86 (CCGTCTAGATGCTGTTATTCATTTTCAAGAAATGG) and JL89 (TCCAGTCCCCCTTTTCTTTTAAAAA). Molecular cloning and DNA sequencing (373A DNA sequencer; Applied Biosystems, Foster City, CA) were done as previously described [4, 17]. The *env* gene sequence and predicted protein sequence of the 6 Thai and 4 US isolates were compared with sequences published in the Los Alamos sequence data bank [1]. Alignment of the sequences was with CLUSTAL V, pairwise nucleotide distances were with DNADIST (PHYLIP 3.5, Kimura two-parameter model), protein distances were with PROTDIST (PHYLIP 3.5, Dayhoff PAM matrix), and the neighbor joining trees were with NEIGHBOR (PHYLIP 3.5) [19]. The chimpanzee simian immunodeficiency virus isolate was used as an outgroup isolate to root the tree (not shown).

**Virus titration and neutralization assays.** To minimize variability between experiments, PBMC from a single donor were cryopreserved and used for all titration and neutralization experiments. The TCID<sub>50</sub> was determined for each virus stock using an assay designed to simulate the neutralization assay. In neutralization experiments, six fourfold dilutions of heat-inactivated plasma ( $4^1$ - $4^6$ ) were aliquoted in quadruplicate in a 96-well plate (40  $\mu\text{L}$  per well). To keep plasma protein concentration constant, dilutions were done in culture media (RPMI 1640 supplemented with 15% fetal calf serum, penicillin, streptomycin, and L-glutamine) containing 25% pooled, heat-inactivated, normal human serum (NHS). Sextuplicate control wells contained medium with 25% NHS. An equal volume of virus stock, representing 100 TCID<sub>50</sub>, was added to each well. After 30 min at  $37^{\circ}\text{C}$ ,  $2 \times 10^5$  PHA-stimulated PBMC were added and incubated overnight at  $37^{\circ}\text{C}$ . Cells were then washed three times by centrifugation in 1-mL cluster tubes (Costar, Cambridge, MA) to remove p24 antigen and plasma anti-p24 antibody. After the final wash, cells were transferred to a 96-well microtiter plate

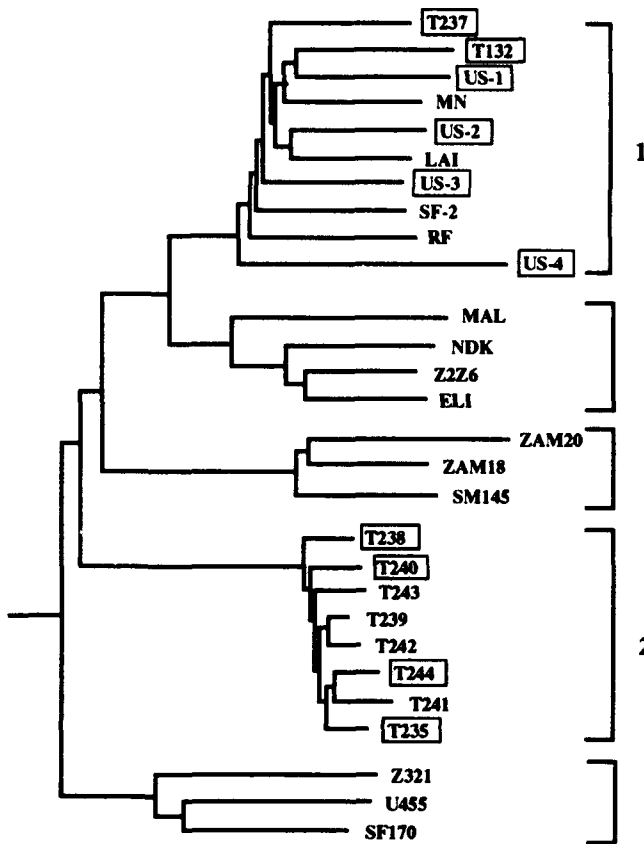
with fresh interleukin-2 medium. Inhibition of PBMC infection was assessed by quantitative p24 measurements of cell supernatants on day 4 by EIA (Coulter, Hialeah, FL). Percentage neutralization (decrease in p24 levels in plasma wells compared with NHS control wells) was plotted against the reciprocal plasma dilution (defined as the dilution of plasma in the presence of virus). The 50% neutralization titer was determined with a curve fit program using the median effect equation (Biosoft, Milltown, NJ) and values were rounded to the nearest 10.

## Results

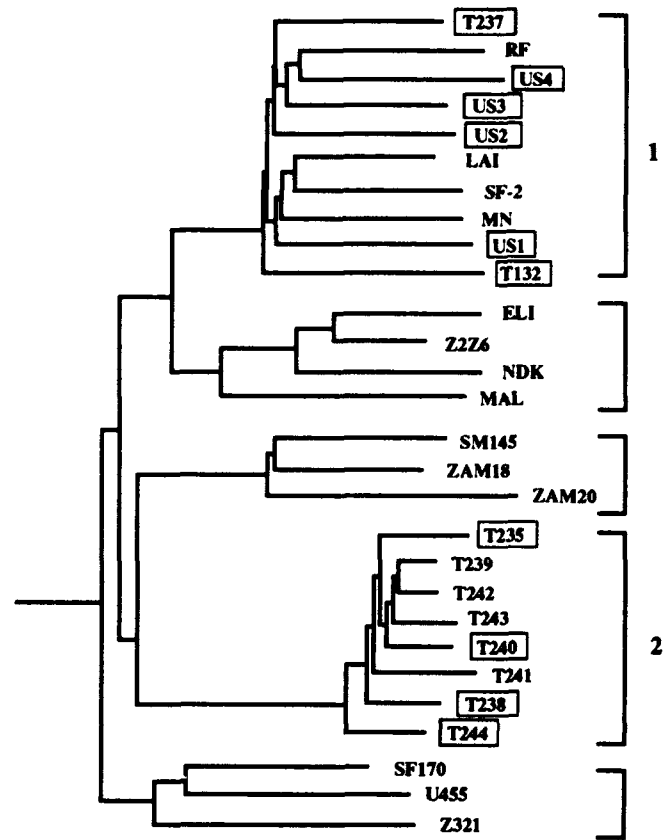
**Genetic characterization of virus isolates.** Because neutralizing antibodies are thought to be directed against epitopes in the envelope glycoprotein (gp160) [20-22], all 10 primary isolates were genetically categorized on the basis of full *env* gene DNA sequence. The neighbor joining trees shown in figure 1 were based on a gp160 DNA and predicted protein sequence alignment for the isolates used in this study and sequences published in the Los Alamos sequence data bank [1]. DNA- and protein-based trees have very similar topology. Five genotypes are easily discerned (vertical bars). The viruses used in this study belonged to the genotypes numbered 1 and 2. Two of the Thai isolates and the 4 US isolates clustered in a single genetic subtype, together with laboratory isolates HIV-1<sub>MN</sub>, HIV-1<sub>LAI</sub>, HIV-1<sub>SF2</sub>, and HIV-1<sub>RF</sub> (genotype 1). The four other Thai viruses clustered closely together in a genetically distinct subtype (genotype 2). Thus, these viruses represent two of the five genetic subtypes depicted in figure 1. On average, viruses in genotypes 1 and 2 differed from each other by 20% on the DNA level and 31% on the protein level.

**Cross-neutralization experiments.** Cross-neutralization assays were done with a panel of 8 plasma specimens obtained from clinically asymptomatic young men. These men were infected with viruses representing the two divergent genetic subtypes; that is, 4 US and 4 Thai plasma specimens each were from donors infected with HIV-1 of genotype 1 and 2 (figure 1) [17]. For genotype 1, plasma samples and virus isolates were obtained from the same individuals. For genotype 2, Thai plasma was not available for some virus isolates expanded for neutralization; thus, plasma and virus isolates are from different individuals. All 8 plasma samples reacted strongly on standard Western blot (Biotek Research Laboratories, Rockville, MD; data not shown). These 8 plasma samples were assayed against the 10 primary isolates of both genotypes and against 3 laboratory strains (HIV-1<sub>IIB</sub>, HIV-1<sub>MN</sub>, and HIV-1<sub>RF</sub>). Each plasma sample was tested against several viruses in two or three independent experiments, and results were similar. Since only  $\sim 30\%$  of HIV-1 strains will infect standard human neoplastic T cell lines [23-25], primary human PBMC were used as target cells in the neutralization assay. Laboratory strains were included in the cross-neutralization analysis because most prior neutralization studies have used these strains. Also, studies with sCD4 (the

## A. DNA Tree



## B. Protein Tree



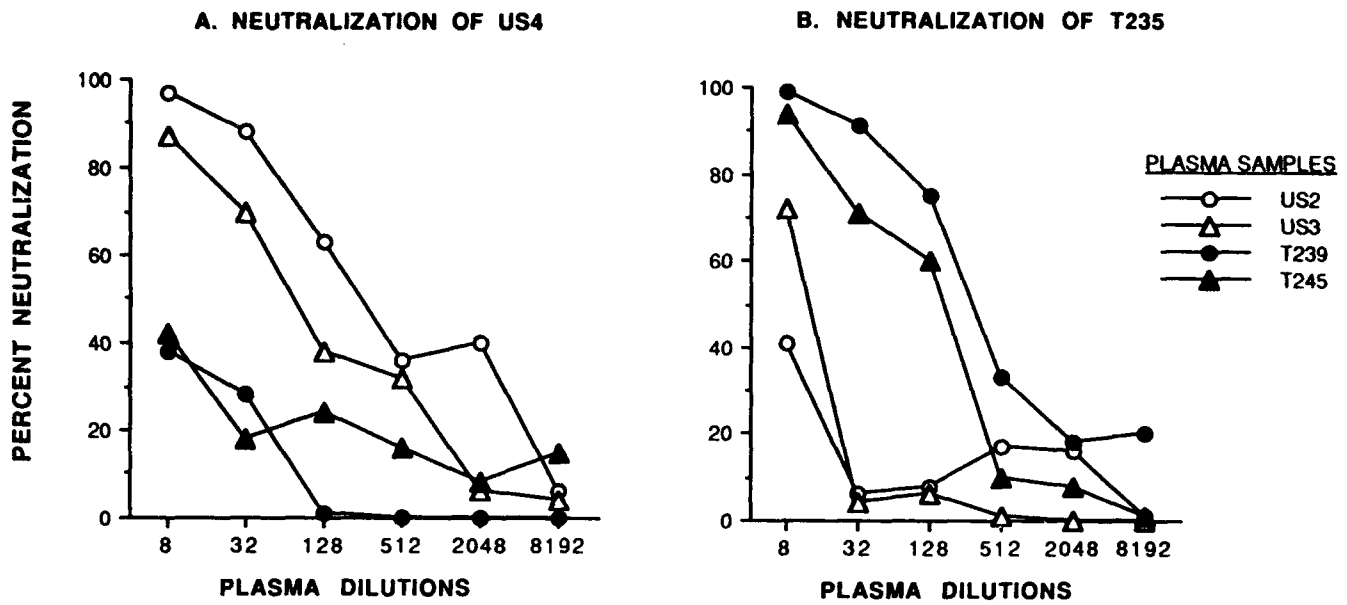
**Figure 1.** Distance trees showing relationship in *env* for HIV-1 isolates. Neighbor joining trees were based on gp160 DNA (A) and predicted protein (B) sequence alignment for isolates used in this study and sequences published in Los Alamos sequence data bank.

soluble form of the cell receptor for HIV-1) have shown that in contrast to primary isolates, laboratory strains are highly sensitive to sCD4 neutralization [26]. The three laboratory strains evaluated belong to genotype 1. Several attempts to generate a genotype 2 laboratory strain by passage of Thai viruses into neoplastic cell lines (H9, HuT-78, CEM) were unsuccessful.

Figure 2 compares neutralization curves for 2 genetically distinct virus isolates (US4 and T235) and 4 plasma samples. Each data point represents the percentage decrease in p24 antigen levels based on the mean value from quadruplicate wells. In these experiments, the median coefficient of variation for p24 antigen production in quadruplicate wells was 21%. The 2 US plasma samples (genotype 1) exhibited strong neutralizing activity against the homotypic US virus but substantially weaker activity against the genotype 2 Thai virus. Likewise, the 2 Thai plasma samples (genotype 2) exhibited significant neutralizing activity only against the genotype 2 Thai virus.

As shown in table 1 and figure 3, 50% neutralization titers for all 8 plasma samples against primary isolates ranged from

<10 to 790. As a group, plasma neutralization titers were significantly higher against viruses of the same genotype. US plasma samples (genotype 1) had higher neutralization titers against US and Thai viruses of genotype 1 (geometric mean, 108 and 250, respectively) than against Thai viruses of genotype 2 (geometric mean, 12). Likewise, Thai plasma samples (genotype 2) reacted more strongly to the 4 Thai viruses of genotype 2 (geometric mean, 96) than against US or Thai genotype 1 viruses (8 and 6, respectively). For each virus isolate, the neutralization profile correlated with the known genotype. Thus, the 2 Thai viruses characterized as genotype 1 had a neutralization profile similar to the US viruses of the same genotype. Between different genotypes, plasma were often nonneutralizing even at the lowest dilution tested (1:8; figure 2). Even with some variability among individual plasma samples, the geometric mean titers from plasma and virus of the same genotype were ~10-fold higher than those obtained from plasma and virus from heterologous genotypes (figure 3). Thus, for the two genotypes of HIV-1 studied, genetic subtype correlated with an antigenically distinct viral serotype.



**Figure 2.** Comparison of neutralization curves for 2 genetically distinct virus isolates and panel of 4 plasma samples. **A**, Genotype 1 virus (US4); **B**, genotype 2 virus (T235). Open symbols are genotype 1 plasma; closed symbols are genotype 2 plasma.

Plasma samples were also assayed against genotype 1 viruses represented by the laboratory strains HIV-1<sub>MN</sub>, HIV-1<sub>III B</sub>, and HIV-1<sub>RF</sub> (table 1). Like the primary HIV-1 isolates, these viruses were more strongly neutralized by genotype 1 plasma. The geometric mean titer of US plasma samples against laboratory strains was 3360 compared with 150 for the heterotypic Thai plasma samples (figure 3). However, in contrast to primary HIV-1 isolates, the laboratory strains were significantly more susceptible to neutralization by plasma samples of both genotypes. The geometric mean titer of US plasma samples against laboratory strains was 3360

compared with 150 against US primary isolates. Likewise, the geometric mean titer for Thai plasma against laboratory strains was 150 compared with 8 and 6, respectively, against US and Thai primary isolates of genotype 1.

**Discussion**

While there is abundant evidence for multiple genetic subtypes of HIV-1, the immunologic significance of divergent genotypes has not been well studied. This report is the first to clearly demonstrate a correlation between HIV-1 genotype

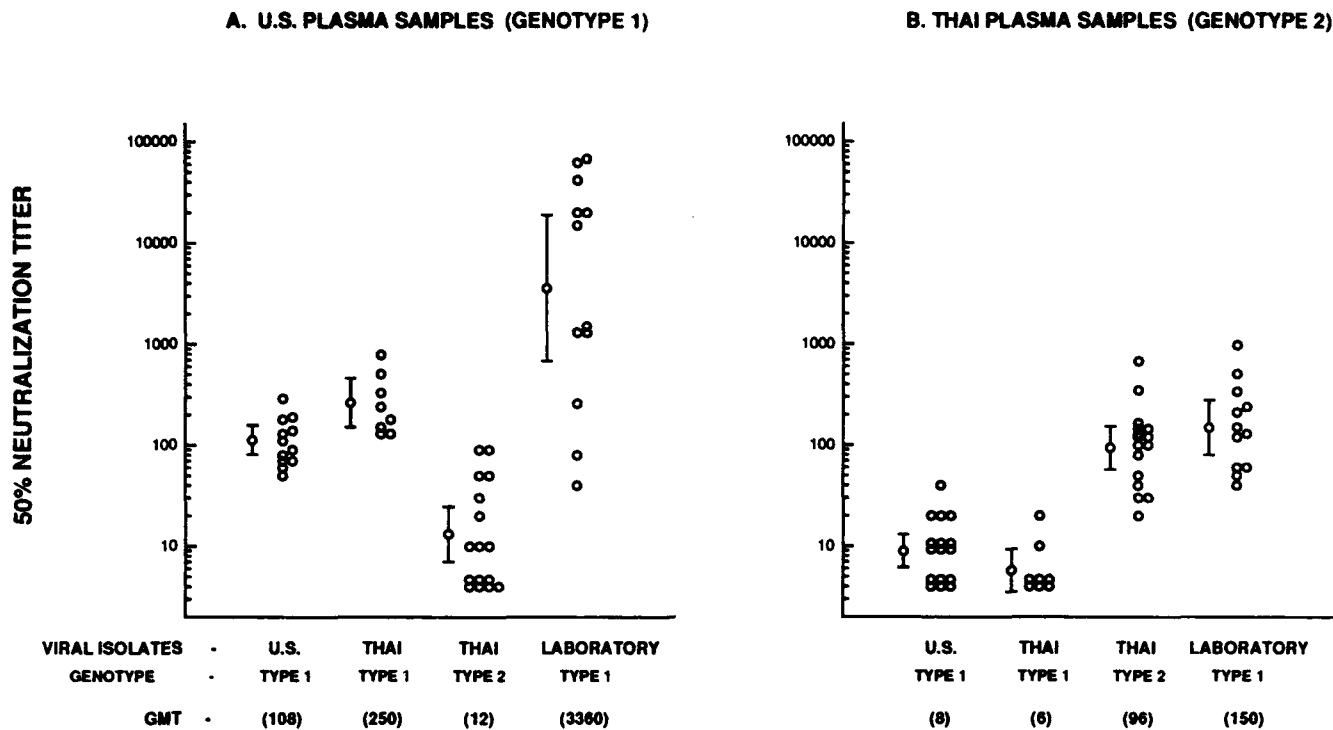
**Table 1.** Cross-neutralization matrix with plasma and virus isolates representing two distinct genetic subtypes.

Human plasma	Thai primary isolates										Laboratory strains, genotype 1		
	US primary isolates, genotype 1				Genotype 1		Genotype 2				MN	III B	RF
	US1	US2	US3	US4	T132	T237	T235	T244	T238	T240			
<b>Genotype 1</b>													
US1	30*	60	110	70	130	240	<8	50	<8	<8	42,000	20,000	40
US2	50	10*	90	290	180	510	<8	50	<8	<8	20,000	260	1400
US3	180	80	50*	70	130	330	10	10	10	<8	62,000	15,000	80
US4	130	140	190	130*	150	790	20	90	90	30	68,000	1300	1300
<b>Genotype 2</b>													
T241	10	<8	10	10	<8	<8	80	130	30	30	120	60	40
T239	40	10	<8	<8	20	<8	680	100	350	160	340	60	50
T245	20	<8	20	<8	<8	<8	110	110	20	100	980	210	240
T235	10	<8	20	10	<8	<8	140†	140	40	50	510	130	150

NOTE. Values represent reciprocal plasma dilution that reduced p24 expression by 50% in phytohemagglutinin-stimulated peripheral blood mononuclear cells.

\* Neutralization with autologous plasma obtained at same time virus was isolated.

† Neutralization with autologous plasma obtained ~ 1 year after virus was isolated.



**Figure 3.** Scatter plot of neutralization titers (shown in table 1) with panel of 4 plasma samples (either US or Thai). Titers from autologous, concomitant plasma and virus pairs are excluded. Error bars indicate geometric mean titer (GMT) and 95% confidence intervals. For calculating GMT, plasma samples that were nonneutralizing at lowest dilution tested (1:8) were assigned values of 1:4.

and antigenic phenotype. The 10 primary HIV-1 isolates we evaluated represent two distinct genetic subtypes as determined by DNA sequence analysis of the full *env* gp160 gene. Using a PBMC-based neutralization assay, we were able to demonstrate that for each virus, neutralization serotype correlated with genetic subtype. In particular, the two Thai viruses of genotype 1 displayed a neutralization phenotype similar to the US viruses of the same genotype. While this relationship between genotype and serotype has been evaluated for only two of the several HIV-1 genotypes, data suggest that it may exist among other subtypes. Cheng-Mayer et al. [27], using a PBMC neutralization assay, showed that several US-derived anti-HIV-1 human sera reacted weakly, or not at all, to 3 virus isolates obtained in Rwanda. Also, other investigators evaluating binding antibody or neutralizing antibody to laboratory strains have shown reduced reactivity between serum and virus or viral peptides from differing geographic locales [11, 21, 28].

Of interest, 1 of the genotype 1 plasma specimens (US4) reacted strongly with 2 of 4 Thai viruses of genotype 2. This may indicate the presence of neutralizing antibodies to a region conserved among HIV-1 subtypes. Delineation of the epitope or epitopes responsible for generating cross-reactive neutralizing antibodies could be an important contribution to development of a broadly effective vaccine. Alternatively, it is possible that cross-reactivity is the result of antibodies to human cellular antigens present on virions. It has been dem-

onstrated recently that human lymphocyte antigens (HLA) are physically associated with HIV-1 virions, and antisera to HLA class I, HLA-DR, and  $\beta$ 2-microglobulin can inhibit infection of cultured cells by HIV-1 [29].

Furthermore, because of presumed molecular mimicry between epitopes of HIV-1 envelope glycoprotein and HLA antigens, sera from some HIV-1-infected persons contain antibodies to HLA class II molecules [30, 31]. Thus, sera from HIV-1-infected persons could theoretically contain anti-HLA antibodies that would neutralize HIV-1. Although PBMC from a single donor were used as target cells in our neutralization assays, the virus stocks were obtained by expansion in PBMC from different donors. Therefore, virus isolates from different genetic or antigenic subtypes could potentially present similar cellular (HLA) antigens that theoretically could be responsible for cross-neutralization. This hypothesis is currently being addressed. Regardless of mechanism, cross-neutralization between genotypes was uncommon in our experiments. Also of note are the relatively low neutralization titers by autologous plasma against concomitantly obtained virus. This is seen in 3 of the 4 subjects for which there were concomitant autologous plasma and virus samples (US1, US2, and US3). This relative neutralization resistance is thought to be a manifestation of "neutralization escape" and has been previously described in lentivirus diseases including HIV infection [32, 33].

The neutralization experiments using laboratory strains

HIV-1<sub>MN</sub>, HIV-1<sub>IIIB</sub>, and HIV-1<sub>RF</sub> demonstrated a marked disparity in the ability of human plasma to neutralize laboratory strains compared with primary isolates of HIV-1. As expected, US plasma neutralized laboratory strains more strongly than did the heterotypic Thai plasma. However, when measured against laboratory strains, all plasma samples displayed a greater magnitude and, importantly, breadth of neutralizing activity. In particular, US plasma samples had neutralization titers 100 to 1000 times higher against HIV-1<sub>MN</sub> than against primary isolates from the United States. This disparity in sensitivity to neutralizing antibody is comparable to neutralization with sCD4, in which primary isolates required 200 to 2700 times more sCD4 than was needed to inhibit laboratory strains [26]. With respect to breadth of reactivity against divergent viruses, the Thai plasma samples (genotype 2) appear to have strong cross-reactivity when assayed against genotype 1 laboratory strains (geometric mean titer, 150) but have little cross-reactivity when assayed against genotype 1 primary isolates (geometric mean titer, 8; figure 3B). Furthermore, the Thai plasma samples give neutralization titers against laboratory strains (150) that are similar to those against homotypic Thai viruses (96). Thus, cross-neutralization analysis using only laboratory strains as representatives of genotype 1 would likely lead to the conclusion that Thai plasma samples have significant cross-reactivity against viruses from a divergent genotype. As previously noted, the strong neutralizing activity of international sera to HIV-1<sub>MN</sub> has been interpreted as evidence for the international predominance of HIV-1 strains antigenically similar to HIV-1<sub>MN</sub>. Our data suggest that laboratory strains (HIV-1<sub>MN</sub> in particular) are hyperneutralizable compared to primary isolates and that the broad neutralizing activity of international plasma or sera against HIV-1<sub>MN</sub> is not representative of plasma reactivity to primary isolates. Furthermore, the ability of a candidate vaccine to induce neutralizing antibody to laboratory strains is likely to overestimate the magnitude and breadth of neutralizing antibody present against primary HIV-1 strains prevalent in a potential vaccine trial population. While the mechanisms for increased susceptibility of laboratory strains to neutralization by sCD4 and antibody may be different [34], it seems apparent that primary and cell culture-adapted laboratory isolates exhibit important biologic differences. A greater focus on primary HIV-1 isolates may be an important component of effective vaccine strategies.

Taken together, these data have important ramifications for HIV vaccine development. As previously noted, currently available glycoprotein subunit vaccines are based on laboratory strains representing a single HIV-1 genotype. In many geographic regions there is already substantial intermixing of genotypes [3], and it is likely that this intermixing will eventually include Europe and North America. The existence of at least five widely distributed genetic subtypes suggests that a globally effective vaccine will have to generate immunity to virus isolates exhibiting a broad range of

genetic and, potentially, antigenic diversity. Our observation that HIV-1 genotypes may represent antigenically distinct serotypes suggests, but does not prove, that immunization with an HIV-1 strain representing one genotype will be ineffective against heterologous subtypes. Such an evaluation can be made only through *in vivo* vaccine challenge studies. However, if inclusion of distinct HIV-1 subtypes is required for the development of a broadly effective vaccine, a more inclusive and systematic effort to characterize the regional prevalence of HIV-1 genotypes and immunotypes may be a critical component of vaccine development. Furthermore, vaccine candidates representing each distinct subtype may have to be constructed and evaluated for breadth of immunogenicity in phase I trials in humans. A broadening of vaccine development efforts to include multiple genotypes or serotypes may reap significant dividends as vaccines face the challenges of HIV-1 diversity.

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