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Quantitative Analysis of Serum Neutralization of Human Immunodeficiency Virus Type 1 from Subtypes A, B, C, D, E, F, and I: Lack of Direct Correlation between Neutralization Serotypes and Genetic Subtypes and Evidence for Prevalent Serum-Dependent Infectivity Enhancement

LEONDIOS G. KOSTRIKIS, YUNZHEN CAO, HELEN NGAI, JOHN P. MOORE, AND DAVID D. HO*

Aaron Diamond AIDS Research Center, New York University School of Medicine, New York, New York 10016

Received 25 July 1995/Accepted 12 October 1995

Human immunodeficiency virus type 1 (HIV-1) M group strains have been assigned to date to nine distinct genetic subtypes, designated A through I, according to phylogenetic analyses of nucleotide sequences of their *env* or *gag* genes. Whether there is any relationship between phylogenetic subtypes and the neutralization serotypes is not clear, yet defining the nature of any such relationship by mathematical means would be of major importance for the development of globally effective HIV-1 vaccines. We have therefore developed a quantitative method to analyze serum neutralization of HIV-1 isolates and to identify HIV-1 neutralization serotypes. This method involves calculations of the neutralization index, $N^{(i)}$, a newly defined parameter derived from plots generated from in vitro neutralization assays, calculations of pairwise serum-virus vector distances, and cluster analyses. We have applied this approach to analyze three independent neutralization matrices involving primary HIV-1 strains and sera from genetic subtypes A, B, C, D, E, F, and I. Detailed serum and HIV-1 isolate cluster analyses have shown that in general, the identified neutralization serotypes do not directly correlate with HIV-1 genetic subtypes. These results suggest that neutralization serotypes developed during natural HIV-1 infection are not governed by antibodies directed against simple epitopes within gp120 monomers. A significant proportion (28%) of 1,213 combinations of sera and HIV-1 isolates caused serum-dependent infectivity enhancement [negative $N^{(i)}$ values] rather than neutralization. We also noted that negative $N^{(i)}$ values tended to correlate better with certain HIV-1 isolates rather than with HIV-1-positive sera. Syncytium-inducing variants of HIV-1 were slightly more likely than non-syncytium-inducing variants to undergo serum-dependent infectivity enhancement, although the latter variants could clearly be susceptible to enhancement.

Human immunodeficiency virus type 1 (HIV-1) M group strains have been assigned to nine genetic subtypes, designated A through I, on the basis of *env* or *gag* gene nucleotide sequences (1, 3–5, 8, 9, 13, 16, 22–26, 29, 32–35, 44–48, 50, 54, 61). Recently, an additional number of HIV-1 recombinants in *env* and *gag* were also identified (51). The existence of HIV-1 variation on this scale has important implications for understanding the global evolution of HIV-1 and for designing antigenically suitable HIV-1 vaccines based on the induction of neutralizing antibodies. However, HIV-1 neutralization depends on the association of antibodies not only with linear epitopes whose structure might vary in a way predictable from knowledge of genetic subtypes, for example epitopes within the V3 loop, but also with discontinuous epitopes within the monomeric and oligomeric forms of the HIV-1 envelope glycoproteins (10, 18, 19, 41, 43, 57). Additional complex epitopes may also be associated with the icosahedral-symmetry organization of the envelope glycoproteins on the viral surface (14, 15, 49). How subtype-dependent sequence variation might affect the structure and presentation of these complex epitopes is not yet possible to predict from our limited knowledge of envelope tertiary and quaternary structure. An additional complexity is that infection-enhancing antibodies or other factors present in the serum may interfere with the effects of neutralizing antibodies. Therefore, it is not obvious that neutralization serotypes should correspond precisely to the genetic subtypes.

It is, however, important to confirm this because if neutralization efficiency is indeed strongly influenced by the degree of genetic heterogeneity predicted by the subtypes (i.e., if the neutralization serotypes are identical to genetic subtypes), an appropriate vaccine strategy might be to design immunogens based on the predominant genetic variants circulating in any one locale at any one time, similar to the current influenza vaccine strategy. In contrast, if all HIV-1 strains are equally susceptible to serum neutralization (i.e., if there is a single neutralization serotype), a single immunogen might suffice worldwide. Alternatively, if HIV-1 isolates or sera are clustered into specific neutralization serotypes (i.e., if the neutralization serotypes do not directly coincide with the genetic subtypes), an appropriate vaccine strategy might be to tailor

* Corresponding author. Mailing address: Aaron Diamond AIDS Research Center, 455 First Ave., New York, NY 10016. Phone: (212) 725-0018. Fax: (212) 725-1126. Electronic mail address: ho@adarc.nyu.edu.

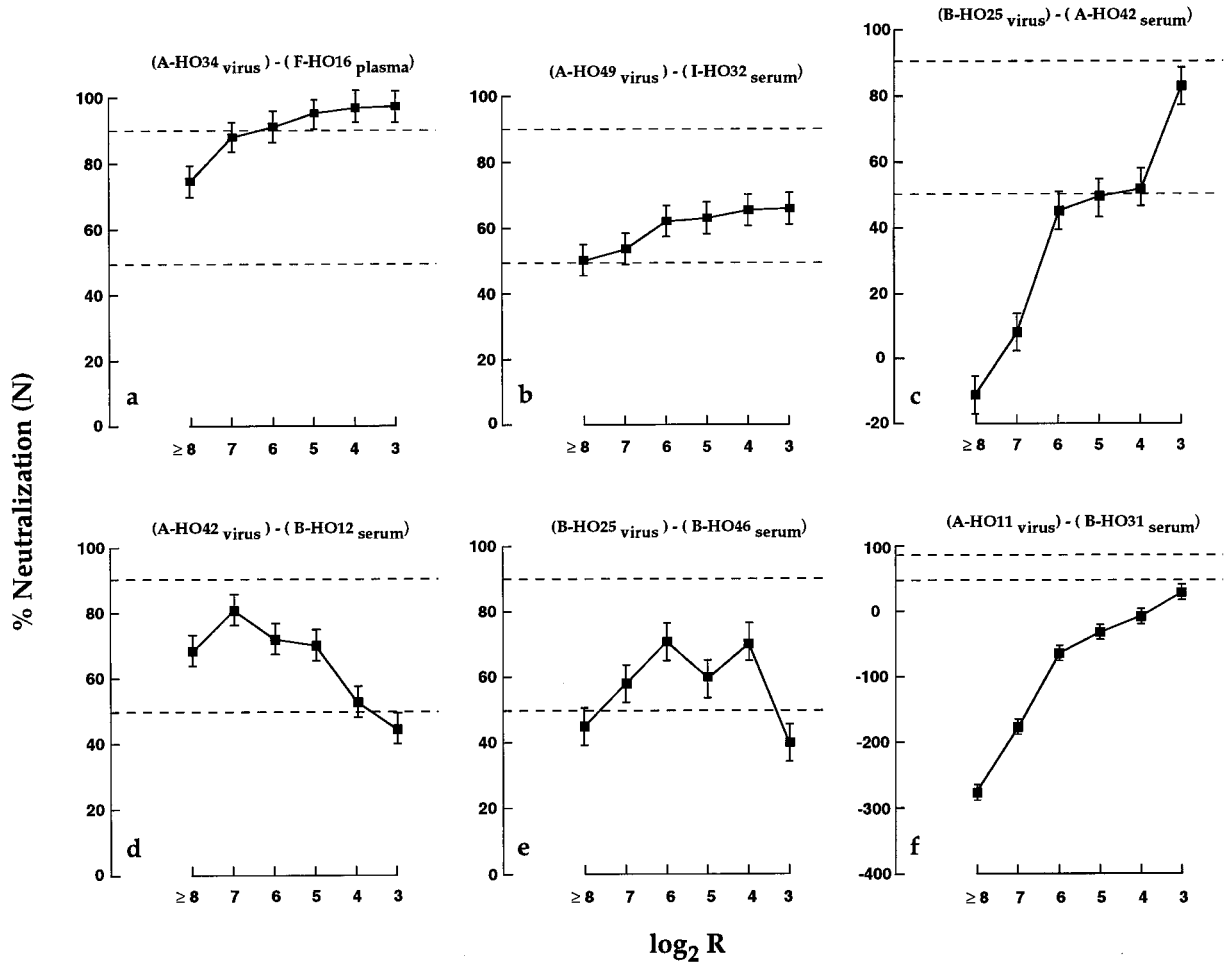

FIG. 1. Six representative serum neutralization plots from in vitro neutralization assays from viral isolates and sera collected from HIV-1-infected patients in Cyprus. The identification number and genetic subtype of the serum and viral isolate used are indicated at the top of each plot. R is the reciprocal of serum dilution. Error bars represent 1 SD of the mean value ($n = 4$). The dashed lines represent the 50 and 90% neutralization levels.

immunogens based on each of the identified neutralization serotypes, irrespective of the genetic subtypes.

To address this issue, we performed in vitro neutralization assays using HIV-1 isolates and sera from the genetic subtypes A, B, C, F, and I and then analyzed the data by using a newly developed method, here referred to as the neutralization index method (NIM). We then used NIM to analyze the results of more extensive neutralization assays using HIV-1 isolates and sera from subtypes A through E (39), with the goal of identifying all putative neutralization serotypes. The results of our analyses revealed that there are a significant number of HIV-1 isolates that exhibit infectivity enhancement rather than neutralization by most sera tested. Cluster analyses revealed that the putative neutralization serotypes do not directly correspond to the genetic subtypes. Furthermore, except for that from Thai subtype E isolates, the analysis of a data set generated by using only sera selected to have high titers of antibodies reactive with monomeric gp120 showed that there can be some correlation between neutralization serotypes and genetic subtypes under certain circumstances. An additional finding was that these sera did not neutralize HIV-1 isolates better than sera with lower titers to monomeric gp120.

MATERIALS AND METHODS

HIV-1 primary isolates and sera. Twenty-four sera and 14 HIV-1 isolates were obtained by the Aaron Diamond AIDS Research Center from patients in Cyprus (30). Descriptions of the virological and clinical profiles of the study subjects from whom HIV-1 was isolated for use in this study, and of the methods used to determine HIV-1 viral subtypes, have been previously reported (30). Other viral isolates and sera analyzed in this study were obtained from repositories organized by the World Health Organization (WHO) and by the National Institute of Allergy and Infectious Diseases (NIAID). Details of these reagents are found in the accompanying report (39).

Serum neutralizing activity against HIV-1 isolates. Twenty-four sera from HIV-1-infected individuals were analyzed for the ability to neutralize a panel of 14 HIV-1 strains isolated from the same patient cohort. The neutralization assay has been described previously (6, 7, 39, 40). It is important to note that all sera were heat inactivated (56°C for 30 min) before their use in the neutralization assays, to destroy complement and other labile proteins. Peripheral blood mononuclear cells used for experimental and control cultures were from the same donor to minimize variation of virus replication in host cells from different donors.

Serum neutralization parameter. The serum neutralization parameter, N, is defined as follows:

$$N = \frac{[p24]_c - [p24]_e}{[p24]_c} \times 100 \quad (1)$$

where [p24] denotes the HIV-1 p24 antigen concentration measured in each culture, c denotes the p24 antigen value in a control culture, and e denotes the corresponding value in the presence of HIV-1-positive patient serum. Therefore,

N can be equal to or less than 100%. Negative values of N arise when $[p24]_e$ is larger than $[p24]_c$ and indicate that virus infection of the culture in the presence of the serum is greater than that of the control. We analyzed a series of plots describing the variation of N with $x = \log_2(R)$, where R is the reciprocal of serum dilution. Experiments are typically carried out for a serum dilution range of $a \leq x \leq b$. Six representative neutralization plots out of a total of 336 from the Cypriot reagents are shown in Fig. 1.

Serum neutralization index. A neutralization index, $N^{(i)}$, is mathematically defined as follows:

$$N^{(i)} = \int_a^b N(x)dx \tag{2}$$

Under experimental conditions, a finite number of datum points is recorded for different discrete values of x. Let these values be $\{x_i\}_{i=1}^{M}$, where $x_i = a$ and $x_M = b$. An approximation to equation 2 is easily obtained by numerical quadratures such as the trapezoidal rule. A more accurate value is obtainable, in theory, by polynomial interpolation and analytical evaluation of the resulting integral. However, because of the magnitude of typical experimental errors in this assay (10 to 20%), the advantage of using such a refined procedure is not clear. Therefore, each $N^{(i)}$ value was calculated by using the trapezoidal rule approximation of equation 2 on the basis of six values, $x = 3, 4, 5, 6, 7$, and 8. Calculations using the trapezoidal rule approximation and the above-specified values of x show that the upper bounds of $N^{(i)}$ is 500.

Twenty-four sera were evaluated for their neutralizing or enhancing activities against a panel of 14 HIV-1 primary isolates representing subtypes A, B, C, F, and I. The neutralization index for each serum-virus assay was computed from the experimental data described above. These values are represented in a matrix $(N_{sv}^{(i)})_{\substack{s=1,24 \\ v=1,14}}$ (see Table 2). Here, we have used matrix notation in which the entry $N_{sv}^{(i)}$ is the neutralization parameter derived from an assay using serum s and virus v. To compare the calculated $N^{(i)}$ values with the more traditional display of ID_{90} and ID_{50} values (highest serum dilutions that produced ≥90 and ≥50% inhibition) from neutralization assays (6, 7, 18), we present in Table 1 matrices of the highest R values that produced $N \geq 50\%$ and $N \geq 90\%$ for each element.

We also calculated the $N^{(i)}$ values from neutralization data from sera and HIV-1 isolates obtained from NIAID and WHO presented as ID_{50} and ID_{90} values in the accompanying report (39). These calculations are presented in matrices $(N_{sv}^{(i)})_{\substack{s=1,19 \\ v=1,13}}$ (Table 3), $(N_{sv}^{(i)})_{\substack{s=1,21 \\ v=1,20}}$ (Table 4), and $(N_{sv}^{(i)})_{\substack{s=1,15 \\ v=1,14}}$ (Table 5). The $N^{(i)}$ values from these matrices were calculated by using the trapezoidal rule approximation from five instead of six values ($x = 3, 4, 5, 6$, and 7), and therefore $N^{(i)} \leq 400$.

Serum and virus neutralization cluster analyses. Our cluster analyses and identification of putative sera neutralization serotypes were based on square matrices, $(N_{sv}^{(i)})$, derived as follows. First, the matrix $(N_{sv}^{(i)})$ was standardized by dividing each column by the magnitude of the largest element in that column. This was done to evaluate the relative neutralizing effects of different sera against tested viruses. We alternatively standardized the matrix $(N_{sv}^{(i)})$ by normalizing each column vector to a unit length. Note that both standardization procedures preserve directions but not necessarily lengths. Next, all pairwise vector distances between rows of the standardized $(N_{sv}^{(i)})$ were computed by two different distance definitions: (i) Euclidean distance

$$d_1(\underline{x}, \underline{y}) = \sqrt{\sum_{k=1}^{14} (x_k - y_k)^2}$$

and (ii) Bray-Curtis coefficient

$$d_2(\underline{x}, \underline{y}) = \sum_{k=1}^{14} |x_k - y_k| / \sum_{k=1}^{14} |x_k + y_k|$$

where $\underline{x}$ and $\underline{y}$ are any two standardized vectors. These scalar distance values are represented in square matrices measuring 24 by 24. For cluster analysis and identification of putative HIV-1 isolates' neutralization serotypes, the original matrix $(N_{sv}^{(i)})$ was reversed (columns were reversed into rows) into matrix $(N_{vs}^{(i)})$. From there, the analysis proceeded as explained above. Similar analyses were also performed on the three additional $N^{(i)}$ matrices (Tables 3 to 5) calculated from neutralization data derived by using reagents obtained by the NIAID and WHO (39).

Algorithms written by us and algorithms implemented in BMDP (11) were used to produce distance matrices. Neutralization serotype trees were constructed by using the single-linkage algorithm from BMDP as well as the neighbor-joining and UPGMA clustering algorithms from PHYLIP (12). Consensus clusters generated from all three clustering algorithms, neighbor joining, UPGMA, and single linkage, were considered putative neutralization serotypes. The identities of neutralization serotypes were investigated by implementing artificial neural network architectures (28, 31).

RESULTS

Neutralizing activities of sera against HIV-1 isolates. In this report, we first describe the neutralizing activities of 24 sera against 14 HIV-1 isolates from subtypes A, B, C, F, and I obtained from HIV-1-infected patients in Cyprus (30). The HIV-1 isolates were derived from subjects at different disease states, from seroconversion to AIDS. There are 4 sera and four HIV-1 isolates from subtype A, 15 sera and eight isolates from subtype B, 1 serum from subtype C, 2 sera and one isolate from subtype F, and 2 sera and one isolate from subtype I.

In Table 1 are presented two values from each neutralization curve, the reciprocals of the traditional ID_{50} and ID_{90} neutralization values, as previously described (6, 7). Additional ID_{50} and ID_{90} neutralization values generated with sera and isolates from subtypes A through E are presented in the accompanying report (39). Consistent with the neutralization data in that report (39), inspection of the ID_{50} and ID_{90} neutralization values derived from assays with the Cypriot samples revealed no obvious subtype-specific patterns of neutralization. Instead, a number of HIV-1 isolates (HO42, HO49, HO04, and HO32) were sensitive to neutralization by most sera, while others (HO43, HO16, HO12, HO11, and HO40) were highly resistant.

We were concerned, however, that drawing definite conclusions from ID_{50} and ID_{90} values alone might be inappropriate. Neutralization curves with uncloned isolates and polyclonal HIV-1^+ sera are often of irregular shape (Fig. 1). Frequently, the curves level off near the 90% neutralization mark, and their flatness can lead to considerable imprecision in the definition of ID_{90} values. This point is exemplified in Fig. 1a, as only a small variation in the extent of neutralization could significantly shift the position at which the experimental curve intercepts the 90% neutralization mark. Neutralization curves resembling that shown in Fig. 1b are very common, in that 50% but not 90% neutralization is achieved. For this type of curve, ID_{50} values may significantly overestimate the extent of neutralization, but the absence of an ID_{90} value underestimates it, especially when the curve levels off just below the 90% neutralization mark. Many neutralization curves are similar to that shown in Fig. 1c; the biphasic nature reflects the polyclonality of neutralizing human sera. The curve shoulder around the ID_{50} mark creates a significant error in the determination of an ID_{50} value, and 90% neutralization is not achieved, yet significant neutralization has clearly taken place. The neutralization curve displayed in Fig. 1d is frequently observed and illustrates another critical point. Decreasing the serum concentration actually increases the extent of neutralization, so that in an extreme case the ID_{50} value could be greater than the ID_{90} value. The type of neutralization profile exemplified by Fig. 1e creates ambiguities in determining the correct ID_{50} value. Finally, some sera cause significant negative neutralization (infectivity enhancement) of certain isolates at most, if not all, dilutions in the range evaluated (Fig. 1f). Nevertheless, inspection of the ID_{50} and ID_{90} values in Table 1 revealed no consistent subtype-specific patterns of neutralization, in that high values were not concentrated along the diagonals.

***N*$^{(i)}$.** Because of the complex factors revealed by the irregular profiles in a large proportion of the 1,213 neutralization curves that were analyzed to generate the four neutralization matrices, we decided to develop a better method to quantitate the neutralization of uncloned primary HIV-1 isolates by polyclonal sera and to identify any underlying neutralization trends

TABLE 1. Neutralization matrix of sera and primary HIV-1 isolates from subtypes A, B, C, F, and I

Source of serum	*R* value for[a]:													
	ID_{50}													
	Primary HIV-1 isolate													
	Subtype A				Subtype B								Subtype F, HO16	Subtype I, HO32
	HO17	HO34	HO42	HO49	HO04	HO11	HO12	HO25	HO27	HO39	HO40	HO43		
Subtype A														
HO17	—	256	256	256	128	—	—	256	64	128	—	—	—	256
HO34	16	128	256	256	128	—	—	256	256	64	—	—	—	256
HO42	32	256	256	128	32	—	—	16	32	64	—	—	—	256
HO49	256	64	256	256	—	—	8	112	—	128	—	—	8	256
Subtype B														
HO04	8	—	256	256	64	32	16	256	256	256	256	—	16	256
HO11	8	—	256	256	256	8	16	256	256	64	16	—	32	256
HO12	—	128	256	256	64	64	16	256	128	256	8	—	—	256
HO25	—	8	256	256	256	8	—	256	256	8	—	—	—	256
HO27	—	256	256	—	256	8	—	128	—	—	—	—	—	256
HO39	—	64	256	128	128	32	—	256	128	256	—	—	—	256
HO40	16	—	256	256	256	16	—	128	256	128	—	—	—	256
HO43	8	256	256	128	128	—	—	—	32	128	—	—	—	256
HO21	—	128	256	256	256	64	16	128	256	256	—	—	—	256
HO28	—	128	—	—	256	—	—	128	—	128	—	—	—	256
HO29	—	256	256	256	128	—	—	128	256	128	—	—	—	256
HO45	8	—	256	256	—	—	—	—	128	256	—	—	—	256
HO46	—	64	256	128	—	—	8	128	128	128	—	—	—	256
HO48	—	256	256	256	256	8	64	256	256	256	32	—	—	256
HO50	16	64	256	256	128	32	8	256	32	32	8	—	—	256
Subtype C,														
HO02	—	16	256	256	256	—	8	256	256	128	32	—	16	256
Subtype F														
HO16[b]	—	256	256	256	32	—	—	—	64	128	—	—	—	128
HO44	—	128	256	64	64	—	—	—	32	32	—	—	—	256
Subtype I														
HO32	—	—	256	256	256	—	—	256	256	64	—	—	—	256
HO31	32	128	255	256	128	—	—	128	256	256	—	—	—	256

[a] Reciprocal of highest serum dilution that produced ≥50% or ≥90% inhibition of in vitro HIV-1 p24 antigen production relative to control cultures. Serum neutralizations with autologous viruses are indicated by boxes. —, no neutralization observed.
[b] Plasma specimen.

which could not be discerned by a simple subjective inspection of the ID_{50} and ID_{90} values. This method, NIM, essentially relies on a newly defined parameter, $N^{(i)}$, which is defined as the area under each neutralization curve and thus makes an allowance for irregularities in curve shapes. As defined, an $N^{(i)}$ value of 500 (or 400 for the matrices displayed in the accompanying report [39]) represents the maximum extent of neutralization that could be achieved, while smaller values indicate a proportionately lesser extent of neutralization. An $N^{(i)}$ value of zero means that the net neutralization from all serum dilutions evaluated is zero. Of particular note is that a negative $N^{(i)}$ value indicates a net enhancement of virus infectivity, exemplified by Fig. 1f. In Fig. 2, the traditional ID_{50} values presented in Table 1 are plotted against the corresponding $N^{(i)}$ values calculated from each neutralization curve. The diversity of neutralization curve profiles is evident by the wide distribution of $N^{(i)}$ values corresponding to a particular serum dilution, showing that an ID_{50} value could reflect a number of different neutralization profiles.

Table 2 displays the $N^{(i)}$ values calculated from the neutralization data derived from the 24 sera and 14 HIV-1 isolates from subtypes A, B, C, F, and I, described initially in Table 1. Inspection of the $N^{(i)}$ values revealed no consistent subtype-specific patterns of neutralization, in that high $N^{(i)}$ values were not concentrated along the diagonal. This finding agrees with the conclusions derived from inspection of the ID_{50} and ID_{90} values in Table 1. However, several underlying neutralization patterns which could not be identified by a simple inspection were detected by cluster analyses of the standardized $N^{(i)}$ values (see Fig. 3; discussed below).

Two HIV-1 isolates, HO11 and HO43, were associated with negative $N^{(i)}$ values with several sera tested (Table 2). These subtype B strains were isolated from two patients who died of AIDS within a year of the blood sampling (30). In contrast, HIV-1 isolates HO34, HO42, HO49, HO04, HO25, HO27, HO39, and HO32 gave relatively high positive $N^{(i)}$ values with most sera. Among the most neutralization-sensitive isolates were HO39, HO49, and HO32.

Tables 3 and 4 display the $N^{(i)}$ values calculated from the neutralization data from 19 sera and 13 HIV-1 isolates from subtypes B, C, and E obtained by NIAID and from 21 sera and 20 HIV-1 isolates from subtypes A through E obtained by WHO. The primary neutralization data are described in the accompanying report (39). In Table 3, HIV-1 isolates 92HT593, 93MW101, and 93TH966 frequently produced positive $N^{(i)}$ values (Table 3), the most neutralization-sensitive isolate being 93MW101. In contrast, nine HIV-1 isolates, 92HT594, 92HT596, 92US711, 91US712, 92US714, 92US715, 93MW960, 93MW099, and 93TH975, gave mostly negative $N^{(i)}$ values; of these isolates, 92HT594, 91US712, and 92HT596 were biologically phe-

TABLE 1—*Continued*

R value for[a]:													
ID_{90}													
Primary HIV-1 isolate													
Subtype A				Subtype B								Subtype F, HO16	Subtype I, HO32
HO17	HO34	HO42	HO49	HO04	HO11	HO12	HO25	HO27	HO39	HO40	HO43		
—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—
16	—	—	—	—	—	—	—	—	—	—	—	8	—
—	—	—	—	—	—	—	—	—	16	—	—	16	64
—	—	—	—	—	—	—	—	—	—	—	—	32	—
—	—	—	—	—	—	—	—	—	8	—	—	—	—
—	—	—	—	—	—	—	—	32	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	8	—	—	—	—	—	—	—	—	—
—	—	—	—	8	—	—	—	—	8	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	32
—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	8
—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	256	—	—	16	—	—	—	—	64	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	16	16
—	—	—	—	—	—	—	—	—	—	—	—	—	8
—	64	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	16	8	—	—	—	—	—	64	16	—	—	—	256

notyped as syncytium-inducing (SI) variants, whereas the other isolates were non-syncytium-inducing (NSI) variants, as determined by an assay of syncytium formation in MT-2 cells (17); the biological phenotype of isolate 93MW099 was not determined. Comparisons of $N^{(i)}$ values between rows revealed no evidence for sera that enhanced infection of most isolates in a serum-specific manner.

In Table 4, neutralization-sensitive isolates include 92UG029, 92TH014, and 92UG005, whereas five HIV-1 isolates, 92BR021, 92BR025, 92UG021, 91UG046, and 92UG024, frequently produced negative $N^{(i)}$ values; of these five isolates, 92UG021, 92UG046, and 92UG024 had the SI phenotype, isolate 92BR021 had the NSI phenotype, and the phenotype of isolate 92BR025 was not determined (62).

Overall, inspection of $N^{(i)}$ values from the three matrices revealed no obvious correlation between high positive $N^{(i)}$ values and individual genetic subtypes. The negative $N^{(i)}$ values are mostly associated with certain HIV-1 isolates of all subtypes, and they do not seem to be directly correlated with sera of any particular subtype. However, there was a moderate correlation between negative $N^{(i)}$ values and biological phenotype, as determined by the ability of isolates to form syncytia in MT-2 cells. Of the 33 HIV-1 isolates obtained from NIAID (Table 3) and WHO (Table 4), 8 were SI variants and 20 were NSI variants, while the phenotypes of 5 isolates are unknown (17, 62). Six of the eight known SI strains (92HT594, 92HT596, 91US712, 92UG021, 92UG046, and 92UG024) more frequently gave negative rather than positive $N^{(i)}$ values with HIV-1-

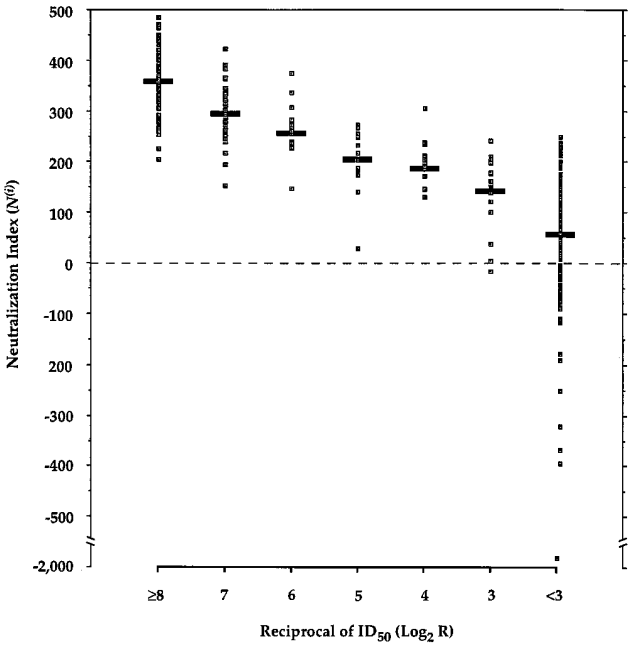

FIG. 2. ID_{50} values derived from viral isolates and sera collected from HIV-1-infected patients in Cyprus (Table 1) plotted against the corresponding $N^{(i)}$ values calculated from the neutralization curves (Table 2). The mean $N^{(i)}$ values calculated from $N^{(i)}$ values from each serum dilution are represented by horizontal lines. Positive $N^{(i)}$ values indicate net neutralization, and negative $N^{(i)}$ values indicate net enhancement.

TABLE 2. $N^{(i)}$ values of sera and HIV-1 isolates representing subtypes A, B, C, F, and I

Source of serum	$N^{(i)}$ for indicated primary HIV-1 isolate[a]													
	Subtype A				Subtype B								Subtype F, HO16	Subtype I, HO32
	HO17	HO34	HO42	HO49	HO04	HO11	HO12	HO25	HO27	HO39	HO40	HO43		
Subtype A														
HO17	112	290	397	385	244	8	45	377	336	272	194	28	112	426
HO34	238	302	359	340	293	**−249**	106	362	317	272	80	26	114	405
HO42	216	205	339	194	254	7	106	186	250	263	101	**−65**	77	403
HO49	386	278	356	328	224	10	121	304	188	328	102	47	241	431
Subtype B														
HO04	152	156	370	374	373	183	202	442	348	407	264	91	209	444
HO11	156	12	360	372	316	3	196	376	360	306	236	35	187	387
HO12	95	251	353	358	254	260	145	403	300	398	197	**−72**	129	413
HO25	123	203	357	334	341	139	30	335	360	197	197	**−59**	127	389
HO27	55	281	269	146	258	**−17**	25	261	175	208	155	**−176**	99	351
HO39	141	236	317	262	325	254	160	361	256	354	94	72	77	348
HO40	191	149	305	277	347	130	63	323	321	365	149	**−46**	135	330
HO43	177	342	316	307	216	**−40**	13	216	252	252	105	7	92	417
HO21	181	279	263	378	286	228	172	345	371	361	232	52	88	436
HO28	74	321	215	215	314	**−80**	**−9**	292	183	321	162	**−84**	164	413
HO29	212	379	373	321	239	64	231	316	344	382	**−63**	83	**−4**	437
HO45	209	109	341	268	226	**−386**	101	142	152	310	116	**−109**	53	362
HO46	52	266	378	277	210	**−181**	101	300	270	335	177	**−76**	117	395
HO48	126	347	374	336	386	37	148	429	356	450	202	63	205	449
HO50	233	257	374	304	282	28	161	332	216	267	178	**−105**	190	421
Subtype C,														
HO02	98	211	358	354	401	1	204	375	365	364	232	13	188	409
Subtype F														
HO16[b]	140	464	314	336	141	1	117	178	265	288	123	11	**−60**	391
HO44	149	311	342	239	227	**−367**	39	69	272	173	145	**−2**	39	385
Subtype I														
HO32	89	211	366	306	286	**−1,890**	29	322	281	282	55	**−66**	7	399
HO31	248	423	252	361	313	**−310**	134	288	470	400	73	58	28	484

[a] Values were calculated as explained in Materials and Methods. Values derived from autologous neutralizations are enclosed by boxes; negative values are in boldface.
[b] Plasma specimen.

positive sera, as did 7 of the 20 known NSI strains (92BR021, 92US711, 92US714, 92US715, 93MW960, 93MW965, and 93TH975). The remaining 2 of the 8 SI strains (92UG029 and 92UG001) frequently produced positive $N^{(i)}$ values, as did the remaining 13 of the 20 known NSI strains (92HT593, 93TH966, 92UG031, 92RW009, 92RW008, 92TH014, 92TH026, 92BR020, 92UG005, 92TH006, 92TH009, 92TH022, and 92TH023) (Tables 3 and 4). Thus, given 28 isolates, of which 8 are SI variants and 20 are NSI variants, the probability of having 6 SI variants out of 13 negative $N^{(i)}$-specific isolates and 2 SI variants out of 15 positive $N^{(i)}$-specific isolates is 0.058 on the basis of Fisher's exact probability test. Therefore, the SI phenotype is correlated with negative $N^{(i)}$-specific isolates. Biological phenotyping on the Cypriot HIV-1 isolates (Table 2) was not done, but the HO11, HO40, and HO43 strains are predicted to be the only SI variants, as judged from the presence of positively charged amino acids at positions 11 (R) and 25 (K) in their V3 loops (30, 62); isolates HO11 and HO43 were two of the three isolates in this panel associated with negative $N^{(i)}$ values (Table 2). Thus, SI strains may be more prone to enhancement of infectivity by sera, although NSI strains clearly also have this property.

Cluster analysis of sera and identification of serum neutralization serotypes. Putative serum neutralization serotypes were identified by cluster analysis on standardized $N^{(i)}$ matrices (Fig. 3A, 4A, 5A, and 6A). In this report, we refer to the cluster constructions as serum neutralization trees and to the final clusters of sera as serum neutralization serotypes. The serum neutralization trees derived by using distance calculations based on either the Euclidean or the Bray-Curtis coefficient were shown to be almost identical, so we present only the neutralization serotype trees based on the Euclidean distance calculations. Figure 3A shows the serum neutralization trees for the Cypriot samples, and Fig. 4A and 5A show the corresponding serum neutralization trees for the NIAID and WHO sera, respectively. Although it is clear that all identified serum neutralization patterns do not correlate with the genetic subtypes, we refrain from making clear-cut partitions of the neutralization trees into a definite number of serotypes because of the relatively limited number of sera and isolates in each matrix. Further analyses similar to those described in this study, including larger matrices of HIV-1 isolates and sera, would be necessary to make definite identifications of HIV-1 neutralization serotypes. Unfortunately, bootstrap values could not be assigned to each of the branch nodes in Fig. 3 to 6 because of the noniterative nature of our quantitative analysis. However, the clusters defined above have been independently confirmed by using iterative artificial neural network architectures (31).

Cluster analysis of HIV-1 isolates and identification of HIV-1 isolate neutralization serotypes. Putative HIV-1 isolate neutralization serotypes were identified by cluster analysis on reverse-standardized $N^{(i)}$ matrices (Fig. 3B, 4B, 5B, and 6B). Similar to the serum neutralization serotype definition, the final clusters of HIV-1 isolates are referred to as HIV-1 isolate neutralization serotypes, and the constructed trees are referred to as HIV-1 isolate neutralization serotype trees. Figure 3B

TABLE 3. $N^{(i)}$ values of sera and HIV-1 isolates representing subtypes B, C, and E

Source of serum	$N^{(i)}$ for indicated primary HIV-1 isolate[a]												
	Subtype B (phenotype)							Subtype C (phenotype)				Subtype E (phenotype)	
	92HT593 (NSI)	92HT594 (SI)	92HT596 (SI)	92US711 (NSI)	91US712 (SI)	92US714 (NSI)	92US715 (NSI)	93MW960 (NSI)	93MW965 (NSI)	93MW101 (ND[b])	93MW099 (ND)	93TH966 (NSI)	93TH975 (NSI)
Subtype B													
92HT593	330	**−58**	65	3	305	**−4**	**−324**	**−14**	**−175**	372	139	149	**−5**
92HT594	231	**−136**	**−34**	**−96**	**−603**	**−182**	**−522**	**−70**	**−219**	346	93	115	**−51**
92HT596	295	**−17**	**−43**	**−70**	43	**−371**	**−340**	**−57**	**−285**	336	**−47**	**−17**	**−119**
92HT599	127	**−3,011**	**−41**	**−69**	**−1,347**	29	315	**−143**	**−215**	293	**−21**	83	40
92US711	140	**−3,269**	350	**−96**	**−743**	68	**−82**	**−118**	395	341	145	198	**−187**
91US712	142	**−3,803**	**−25**	**−96**	**−890**	**−36**	**−834**	**−45**	400	303	**−120**	192	**−187**
92US714	179	**−3,647**	**−35**	90	292	**−225**	**−875**	**−68**	393	293	**−106**	43	**−176**
92US715	147	**−3,770**	**−30**	**−27**	**−216**	**−194**	**−700**	**−141**	384	233	**−211**	61	**−244**
92US716	132	**−3,557**	**−45**	**−90**	**−287**	**−21**	**−675**	**−66**	390	215	233	108	**−167**
Subtype C													
93MW959	226	**−3,129**	354	**−96**	**−1,373**	100	**−35**	**−25**	394	287	111	193	**−130**
93MW960	79	**−1,424**	226	**−96**	**−1,372**	**−5**	**−285**	**−4**	385	347	264	60	**−135**
93MW965	**−1,155**	**−3,276**	**−45**	**−67**	**−464**	**−46**	364	11	397	277	78	80	**−124**
93MW101	**−2,700**	**−2,843**	245	293	367	**−720**	**−1,111**	27	**−228**	254	118	86	**−200**
93MW099	**−806**	**−3,559**	**−44**	**−78**	**−76**	**−476**	**−629**	39	**−160**	0	72	196	**−160**
Subtype E													
93TH966	**−2,214**	**−3,741**	368	**−87**	**−394**	**−426**	**−274**	45	12	317	65	117	**−240**
93TH975	**−85**	**−2,024**	224	**−91**	**−181**	**−621**	**−827**	77	**−28**	236	**−159**	238	**−30**
93TH976	125	**−1,882**	268	**−95**	**−485**	**−136**	**−918**	70	**−116**	263	**−239**	193	99
93TH301	137	**−3,071**	**−45**	69	130	**−238**	**−1,160**	**−116**	**−216**	138	**−286**	**−21**	**−180**
92TH302	165	**−3,165**	**−45**	**−96**	**−788**	**−204**	**−620**	**−68**	**−285**	248	**−308**	**−334**	**−363**

[a] Values were calculated from neutralization data presented in Tables 3 and 4 of the accompanying report (39). Values derived from autologous neutralizations are enclosed by boxes; negative values are in boldface. HIV-1 isolates and sera were obtained by NIAID.
[b] ND, phenotype not determined.

shows the HIV-1 isolate neutralization trees for the Cypriot samples; Fig. 4B and 5B show the corresponding HIV-1 isolate neutralization trees for the NIAID and WHO isolates, respectively.

Analysis of selected sera and HIV-1 isolates with high anti-gp120 monomer titers. In Table 5, we present the $N^{(i)}$ values derived from sera that were selected from each subtype on the basis of high binding titers to gp120 monomers from the same subtype (39). To assess the effect on neutralization dictated by the selection of sera with high anti-gp120 monomer titers, we calculated and compared the mean $N^{(i)}$ values and the percentage of negative $N^{(i)}$ values from each of the four $N^{(i)}$ matrices. Furthermore, we produced two equivalent versions of a matrix by selecting random pairs from the full set of $N^{(i)}$ values in Tables 3 to 5 by using a pseudorandom number generator algorithm (63).

The mean $N^{(i)}$ value calculated from the high anti-gp120 titer-selected matrix, a total of 210 elements (Table 5), is 22 (standard deviation [SD] = 205), and the percentage of $N^{(i)}$ values that were negative is 40. The mean $N^{(i)}$ value from the original three unselected $N^{(i)}$ matrices (Tables 2 to 4)—a total of 1,003 elements—is 43 (SD = 492), and the percentage of negative $N^{(i)}$ values is 28. It is evident, however, that the large SD value is a result of the high negative $N^{(i)}$ values derived from HIV-1 isolate 92HT594 [mean $N^{(i)}$ is −2,599, n = 19] (Table 3). The mean $N^{(i)}$ value calculated from the same three $N^{(i)}$ matrices after excluding the $N^{(i)}$ outlier values derived from this isolate—a total of 984 elements—is 94 (SD = 280), and the percentage of negative $N^{(i)}$ values is 26. The equivalent mean $N^{(i)}$ value calculated from the WHO and NIAID $N^{(i)}$ matrices only [a total of 648 elements by excluding the $N^{(i)}$ outlier values from 92HT594 isolate] is 34 (SD = 300), and the percentage of negative $N^{(i)}$ values is 37. Furthermore, the mean $N^{(i)}$ values from two matrices created by selecting random pairs from the full set of $N^{(i)}$ values are 65 (SD = 340) and 62 (SD = 362), and the percentages of negative $N^{(i)}$ values are 28 and 30, respectively. Thus, selecting a panel of sera on the basis of high anti-gp120 monomer antibody titers failed to identify sera with statistically significant higher than average neutralization activity and lower than average infection-enhancing activity; if anything, the converse was observed. To investigate whether there was any inverse correlation between anti-gp120 monomer antibody titers and $N^{(i)}$ values, we plotted the midpoint anti-gp120 titer values (39) against the calculated homotypic $N^{(i)}$ values but found no statistically significant correlation relating the two parameters (data not shown).

Phenetic patterns of V3 loop amino acid sequences do not correlate with neutralization serotypes. Phenetic analyses of the amino acid sequences of the V3 loops from 27 Cypriot sequences (30) were performed by using a structure-based amino acid substitution matrix as previously described (29, 56, 61). Inspection of the generated phenogram (data not shown) revealed that phenetic clusters do not correlate with the neutralization serotypes. Similar phenograms were also constructed for the WHO samples (61). A direct correlation between the HIV-1 isolate neutralization serotypes, phenetic patterns of the V3 loop, and genetic subtypes (61) was observed only for the five subtype E HIV-1 strains (92TH006, 92TH023, 92TH009, 92TH022, and 92TH024) from Thailand (Fig. 5B).

DISCUSSION

Our interest in developing NIM to analyze the neutralizing activities of sera against primary HIV-1 isolates from infect-

TABLE 4. $N^{(i)}$ values of sera and HIV-1 isolates representing subtypes A, B, C, D, and E

Source of serum	$N^{(i)}$ for indicated primary HIV-1 isolate[a]									
	Subtype A (phenotype)					Subtype B (phenotype)				Subtype C, 92BR025 (ND)
	92UG029 (SI)	92UG031 (NSI)	92UG037 (ND[b])	92RW009 (NSI)	92RW008 (NSI)	92TH014 (NSI)	92TH026 (NSI)	92BR020 (NSI)	92BR021 (NSI)	
Subtype A										
92UG029	50	122	**−197**	118	94	237	159	158	59	**−92**
92UG031	380	246	251	255	244	126	140	150	**−110**	149
92UG037	83	318	290	283	250	256	64	**−96**	**−230**	**−79**
92RW009	142	143	117	95	384	205	**−15**	18	2	**−295**
92RW008	329	326	383	385	222	333	276	273	224	377
Subtype B										
92TH014	314	135	1	120	219	258	103	1	168	**−70**
92TH026	343	188	71	170	124	311	7	18	**−79**	14
92BR020	163	104	23	**−169**	212	224	16	**−41**	93	**−46**
92BR021	32	**−46**	**−63**	**−508**	139	225	**−96**	**−101**	**−23**	**−204**
92BR023	289	193	266	243	240	268	122	19	182	101
Subtype C, 92BR025	329	166	261	249	200	224	88	**−87**	194	**−55**
Subtype D										
92UG001	364	121	**−364**	**−39**	253	90	141	32	**−49**	**−21**
92UG005	370	163	235	259	220	316	299	229	198	16
92UG021	293	196	**−64**	**−65**	297	193	15	127	**−304**	**−68**
92UG046	202	63	**−142**	**−50**	284	216	**−105**	**−183**	**−368**	**−88**
92UG024	379	59	**−226**	**−160**	128	183	77	4	**−170**	**−27**
Subtype E										
92TH006	333	60	50	**−96**	**−180**	109	**−112**	**−44**	183	**−345**
92TH009	8	231	265	95	153	155	**−23**	**−23**	136	**−18**
92TH022	319	322	357	205	171	260	10	**−105**	**−44**	**−255**
92TH024	239	127	115	81	99	230	58	107	203	6
92TH023	346	**−28**	98	3	62	190	**−130**	**−77**	**−38**	**−129**

[a] Values were calculated from neutralization data presented in Tables 1 and 2 of the accompanying report (39). Values derived from autologous neutralizations are enclosed by boxes; negative values are in boldface. HIV-1 isolates and sera were obtained by WHO.

[b] ND, phenotype not determined.

ed patients derives from our concern to define the neutralization serotypes of HIV-1 by mathematical means and to understand their relationships with the genetic subtypes. Identification of neutralization serotypes of HIV-1 would have important strategic implications for the development of globally effective HIV-1 vaccines. We began our analysis with calculations of the neutralization index, $N^{(i)}$, a new parameter that we introduced in this report, which is mathematically defined as the area under the neutralization curve bounded by the lowest and the highest serum dilution measurements. Subsequently, we derived all pairwise serum–HIV-1 isolate vector distances from standardized $N^{(i)}$ values, and we performed cluster analyses. The interpretation of neutralization data based on the neutralization index calculation allows the entire data set used for generation of a serum neutralization curve to be taken into account irrespective of the shape of that curve and clearly reveals when infectivity enhancement has occurred. In contrast, recording only ID_{50} and ID_{90} values obscures any evidence for negative neutralization, or infectivity enhancement, which occurs with HIV-1-positive sera and certain isolates. Additionally, recording only two points from the serum neutralization curves generates numerous null values in the final matrices (Table 1), which thwarts further quantitative analyses to identify any underlying neutralization patterns.

Using NIM to analyze the neutralization data presented in this and the accompanying report (39) revealed that the identified neutralization serotypes—serum and HIV-1 isolate neutralization serotypes—do not generally correspond directly to the genetic subtypes. The lack of a direct association between neutralization serotypes and the genetic subtypes suggests that antibodies to continuous epitopes on gp120, such as those on the V3 loop, are unlikely to be major contributors to the cross-neutralization of primary isolates that can be observed at low levels with some HIV-1-positive sera. If they were, one would expect a direct correlation between the derived neutralization serotypes and either the genetic subtypes or the identified phenetic patterns of the V3 loop; such correlations were not generally observed. Our conclusions concerning neutralization serotypes are also consistent with the observation that strong, subtype-independent neutralization of primary HIV-1 strains can be caused by a number of neutralizing human monoclonal antibodies (60). A direct correlation between the HIV-1 isolate neutralization serotypes and genetic subtypes was observed only for the five subtype E HIV-1 strains (92TH006, 92TH023, 92TH009, 92TH022, and 92TH024) from Thailand (Fig. 5B) (61). The V3 amino acid sequences from these strains were found to cluster together by means of phenetic analysis (61). However, the entire gp120 molecules from Thai subtype E isolates are also genetically (61) and antigenically (42) homogeneous, so the significance of the V3 loop clustering for understanding isolate neutralization subtypes is not absolutely clear. The clustering of Thai subtype E strains as a single neutralization serotype supports the use of immunogens based on the E, rather than B, subtype for vaccine trials in Thailand.

A loose correlation between the genetic subtypes and serum neutralization serotypes was also observed with most sera that were selected for their high anti-gp120 monomer antibody titers (Fig. 6A), although the potencies of the observed neutralizations were not correlated with the anti-gp120 antibody

TABLE 4—*Continued*

$N^{(i)}$ for indicated primary HIV-1 isolate[a]									
Subtype D (phenotype)					Subtype E (phenotype)				
92UG001 (SI)	92UG005 (NSI)	92UG021 (SI)	92UG046 (SI)	92UG024 (SI)	92TH006 (NSI)	92TH009 (NSI)	92TH022 (NSI)	92TH024 (ND)	92TH023 (NSI)
106	147	2	71	**−88**	**−35**	57	89	**−211**	**−8**
386	299	**−7**	55	**−153**	69	167	252	29	109
86	349	**−197**	54	28	338	268	344	241	274
76	289	**−128**	**−32**	63	379	366	367	373	315
312	389	88	90	209	145	141	202	**−33**	118
253	278	60	**−114**	**−204**	383	386	356	334	337
377	295	45	**−35**	**−562**	144	205	241	**−557**	46
54	166	**−142**	**−21**	**−267**	263	120	323	248	147
−92	26	22	**−70**	**−1,216**	**−150**	**−146**	112	**−224**	**−20**
340	335	**−39**	**−68**	**−26**	**−78**	**−210**	**−283**	**−129**	**−135**
338	334	**−61**	**−70**	**−70**	329	344	282	129	194
378	218	85	73	**−124**	**−65**	24	142	**−57**	**−237**
384	355	13	293	135	185	127	304	212	98
209	120	**−65**	56	**−71**	333	319	357	310	282
165	100	**−154**	10	**−3**	363	304	356	248	294
338	52	56	26	**−278**	197	179	226	63	125
287	**−28**	**−96**	**−103**	**−94**	341	276	293	120	212
132	256	**−398**	**−118**	**−148**	**−189**	215	177	**−202**	61
376	355	**−61**	**−130**	**−245**	**−92**	192	159	180	**−69**
393	205	397	**−120**	**−229**	**−59**	68	**−133**	**−87**	**−228**
381	154	82	**−154**	**−1,035**	386	389	355	383	377

titers. It may be that choosing sera on the basis of particularly high anti-gp120 titers selects for sera rich in antibodies to continuous epitopes such as the V3 loop, but this procedure does not necessarily identify the most strongly neutralizing sera. Overall, our current interpretation is that identified neutralization serotypes are more likely to be influenced by antibodies to complex epitopes, perhaps dependent on the conformation of the envelope glycoproteins in their native form, rather than by linear epitopes. Linear epitopes, such as those on the V3 loop, may significantly contribute to neutralization under certain circumstances, for example with the Thai subtype E isolates or with sera selected for high anti-gp120 antibody titers, but we found no indication that whenever there might be a dominant contribution of V3-directed antibodies to neutralizing activity, this was in any way associated with the strength of that activity.

One of the most surprising features revealed by NIM was that a significant proportion (28% of 1,003) of sera and HIV-1 isolate combinations from all genetic subtypes caused infectivity enhancement rather than neutralization. The magnitude of enhancement was variable, but mostly in the same range as the magnitude of neutralization. Our definition of infectivity enhancement in this study is that a serum-virus combination produces a net negative $N^{(i)}$ value. However, it should be noted that enhancing antibodies counter the effect of neutralizing ones, so relying only on net enhancement [negative $N^{(i)}$ values] to assess the prevalence of enhancing antibodies underestimates their occurrence. It may be more common for enhancing antibodies to only partially counteract neutralizing ones, leading to a reduction in the extent of neutralization that might otherwise have occurred. Sera selected to contain high titers of antibodies reactive with gp120 monomers were, if anything, more likely to cause infectivity enhancement than were unselected sera. Furthermore, we observed no correlation between anti-gp120 antibody titers and the $N^{(i)}$ value, a measure of neutralization potency. Our current interpretation of these results is that neither the neutralizing nor the enhancing activity is governed by antibodies directed against gp120 monomers. Alternatively, the serum neutralizing and enhancing infectivity activities may be both gp120 monomer specific but may cancel each other out, wholly or in part, so that no statistically significant net effect is observed.

Enhancing antibodies have been previously demonstrated in vitro in sera from HIV-1-infected patients (20, 21, 36, 37, 52, 53, 59). However, these studies have relied mostly on T-cell line-adapted HIV-1 strains, and there have been few detailed studies on primary virus neutralization by HIV-1-positive sera. Three mechanisms of infectivity enhancement have been documented: Fc-mediated absorption of immune complexes onto the surfaces of target cells (21, 59), an antibody-dependent, complement-mediated enhancement (36, 37), and a gp120 antibody-specific triggering of the HIV-cell fusion reaction (27, 55, 58). The first two mechanisms tend to be mediated most efficiently by antibodies to the ectodomain of the transmembrane glycoprotein gp41, whereas the third mechanism has been shown to be mediated by monoclonal antibodies to the V3 loop and CD4-binding-site-related epitopes on gp120 (27, 55, 58). Mechanistically, this process may resemble soluble CD4-activated fusion, described first for simian immunodeficiency virus and HIV-2 (2, 38). Our observation that sera rich in anti-gp120 antibodies are liable to cause infectivity enhancement may be significant in the context of the anti-gp120 monoclonal antibody-induced fusion mechanism, especially when it is considered that antibodies to both the V3 loop and CD4-binding-site epitopes are highly prevalent in HIV-1-positive sera (41). The potential contributions of the other

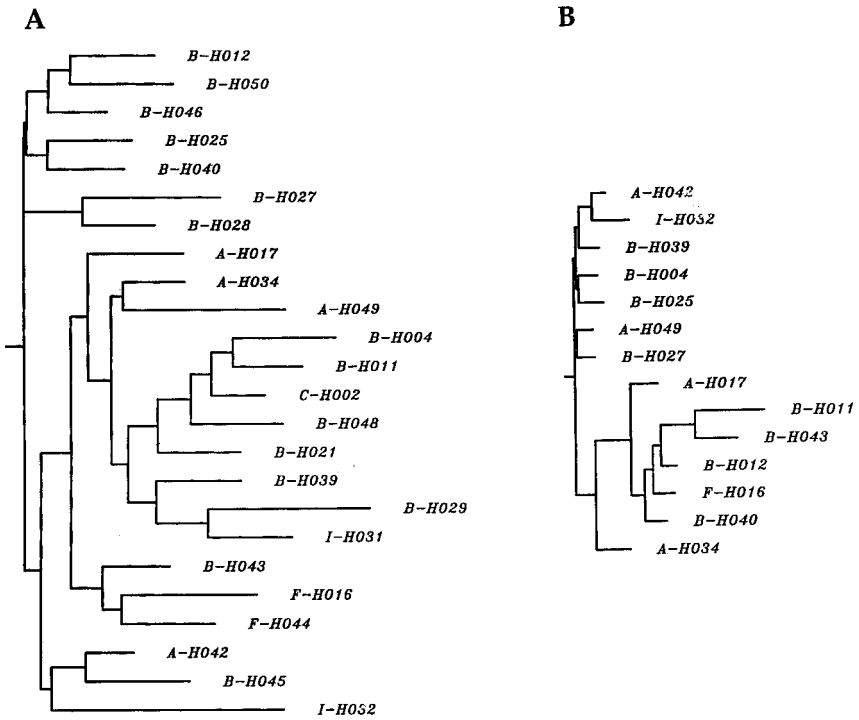

FIG. 3. Serum neutralization tree for 24 sera (A) and HIV-1 isolate neutralization tree for 14 HIV-1 isolates (B) from subtypes A, B, C, F, and I. The neutralization serotype relationships were determined on the basis of the calculated pairwise Euclidean distances between vectors from the standardized matrix ($N_{sv}^{(i)}$) (Table 2) by using the neighbor-joining clustering algorithm.

known enhancement mechanisms should not be discounted, since sera with high anti-gp120 titers may also have relatively abundant antibodies to the gp41 epitopes associated with complement- or Fc-mediated enhancement. However, under the conditions of our assays, complement-mediated enhancement is unlikely, as all sera were heated to 56°C for 30 min prior to use.

It is evident from the calculated $N^{(i)}$ values (Tables 2 to 4) and from the identified HIV-1 isolates' neutralization sero-

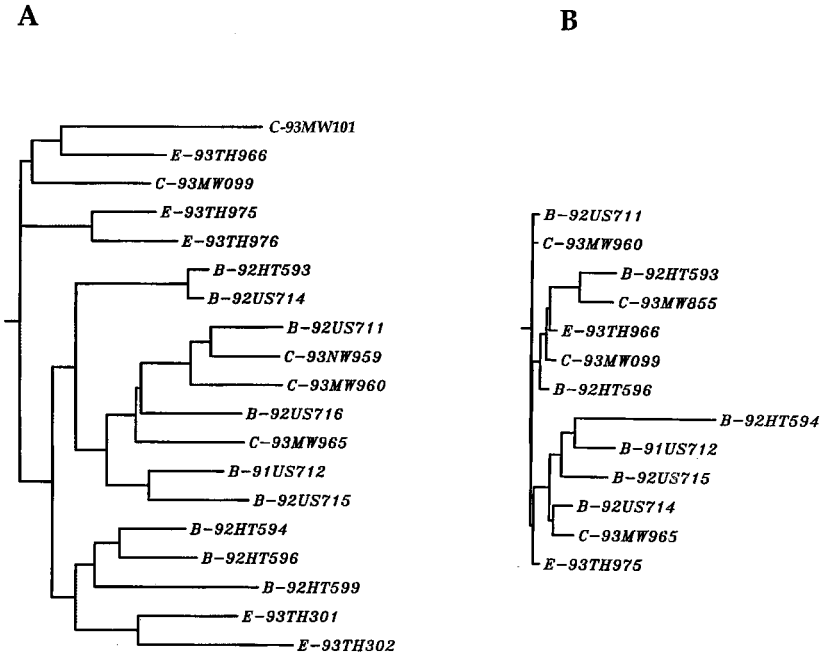

FIG. 4. Serum neutralization tree for 19 sera (A) and HIV-1 isolate neutralization tree for 13 HIV-1 isolates (B) from subtypes B, C, and E. The neutralization serotype relationships were determined on the basis of the calculated pairwise Euclidean distances between vectors from the standardized matrix ($N_{sv}^{(i)}$) (Table 3) by using the neighbor-joining clustering algorithm.

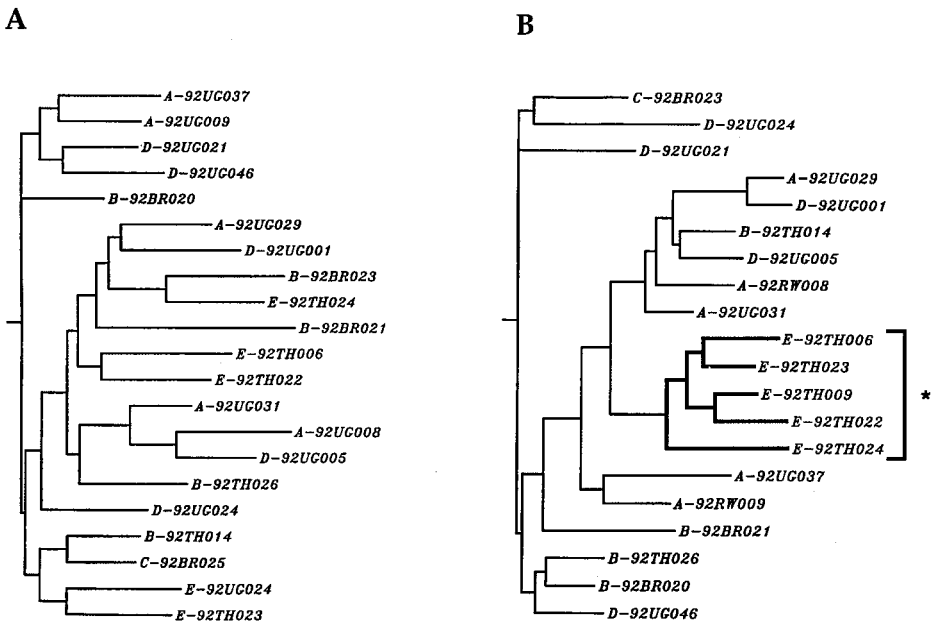

FIG. 5. Serum neutralization tree for 21 sera (A) and HIV-1 isolate neutralization tree for 20 HIV-1 isolates (B) from subtypes A through E. The neutralization serotype relationships were determined on the basis of the calculated pairwise Euclidean distances between vectors from the standardized matrix ($N_{sv}^{(i)}$) (Table 4) by using the neighbor-joining clustering algorithm. The bracket and asterisk on the right in panel B denote an HIV-1 neutralization serotype formed by the five subtype E HIV-1 strains (92TH006, 92TH023, 92TH009, 92TH022, and 92TH024) from Thailand.

types (Fig. 3B, 4B, and 5B) that the negative $N^{(i)}$ values tend to be isolate and not serum specific. Our analysis showed that although the infectivities of some of these isolates (e.g., 92HT594 and 93TH975) were enhanced by almost all sera tested, several sera (e.g., 92US711 and 93MW965) that enhanced the infectivities of these isolates also neutralized other isolates. This phenomenon parallels what has been observed with human monoclonal antibodies (27, 55, 58). Thus, the nature of the virus isolate may determine whether a particular serum or monoclonal antibody is enhancing or neutralizing. Furthermore, in preliminary studies, we have occasionally observed that sera negative for HIV-1 antibodies can also enhance the infectivities of certain isolates. Thus, a portion of the observed infectivity enhancement by HIV-1-positive sera may be due to other, as yet undefined mechanisms associated with some non-HIV-1-specific factor(s) (64). Nevertheless, these results suggest that serum-dependent infectivity enhancement may influence HIV-1 pathogenesis in vivo.

In summary, we have introduced a new quantitative method, NIM, to analyze the neutralizing or enhancing activities of sera with HIV-1 isolates by mathematical means, and we have shown that the putative neutralization serotypes—serum and isolate neutralization serotypes—are not identical and presently cannot be predicted by knowledge of the genetic subtypes alone. We are not certain about the exact nature of the neutralizing antibodies that govern the identified neutralization serotypes, but we have shown that neutralizing antibodies directed against linear epitopes on gp120 monomers are un-

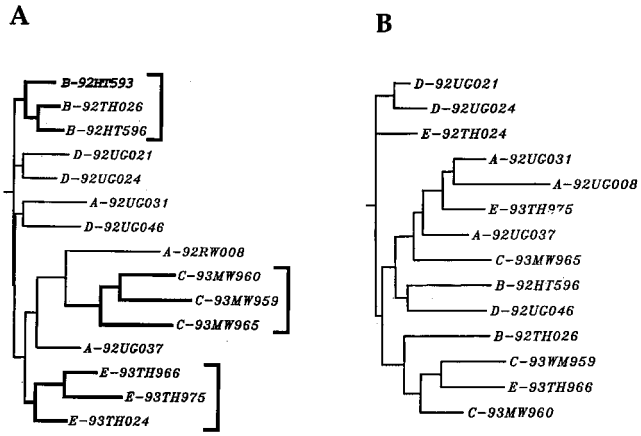

FIG. 6. (A) Serum neutralization tree for 15 sera from subtypes A through E (Tables 3 and 4) selected to have high titers of antibodies reactive with monomeric gp120 from the same subtype. (B) HIV-1 isolate neutralization tree for 14 HIV-1 isolates from subtypes A through E. The neutralization serotype relationships were determined on the basis of the calculated pairwise Euclidean distances between vectors from the standardized matrix ($N_{sv}^{(i)}$) (Table 5) by using the neighbor-joining clustering algorithm. The brackets on the right in panel A denote three serum neutralization serotypes formed by three subtype B sera (92HT593, 92TH026, and 92HT596), two from Haiti and one from Thailand, three subtype C sera (93MW960, 93MW959, and 93MW965) from Malawi, and three subtype E sera (92TH966, 92TH975, and 92TH024) from Thailand.

TABLE 5. $N^{(i)}$ values of sera from subtypes A, B, C, D, and E selected to have high anti-gp120 monomer titers

Source of serum	$N^{(i)}$ for indicated primary HIV-1 isolate[a]													
	Subtype A (phenotype)			Subtype B (phenotype)		Subtype C (phenotype)			Subtype D (phenotype)			Subtype E (phenotype)		
	92UG031 (NSI)	92RW008 (ND[b])	92UG037 (ND)	92HT596 (SI)	92TH026 (NSI)	93MW959 (NSI)	93MW960 (NSI)	93MW965 (NSI)	92UG021 (SI)	92UG024 (SI)	92UG046 (SI)	92TH024 (ND)	93TH966 (NSI)	93TH975 (NSI)
Subtype A														
92UG031	8	**−90**	**−110**	42	**−389**	**−3**	**−11**	69	60	40	**−20**	177	247	**−4**
92RW008	218	224	143	13	**−37**	458	142	424	14	**−25**	209	351	456	95
92UG037	42	**−103**	**−150**	**−322**	229	251	83	124	22	76	52	80	147	170
Subtype B														
92HT593	**−214**	**−7**	**−274**	47	111	71	12	**−22**	67	86	201	141	202	**−157**
92TH026	**−141**	**−33**	**−453**	168	52	38	17	**−105**	109	51	**−78**	102	121	**−208**
92HT596	**−107**	47	**−466**	**−4**	120	**−33**	81	**−29**	163	34	**−45**	145	155	**−172**
Subtype C														
93MW960	64	8	58	7	**−56**	436	246	421	**−207**	**−285**	**−581**	**−97**	363	**−86**
93MW965	**−17**	114	**−238**	**−692**	19	**−56**	101	366	**−172**	**−280**	**−417**	**−57**	244	**−383**
93MW959	251	74	287	**−413**	316	449	465	200	**−100**	**−269**	**−532**	**−166**	397	**−55**
Subtype D														
92UG046	**−314**	**−101**	**−200**	**−363**	**−96**	**−32**	**−168**	**−24**	130	207	**−11**	75	135	**−44**
92UG021	67	**−49**	**−100**	92	76	**−202**	119	189	129	163	77	63	29	**−12**
92UG024	**−63**	**−87**	**−361**	66	95	56	70	145	70	156	55	33	**−35**	**−201**
Subtype E														
93TH966	**−204**	**−176**	**−337**	102	347	375	227	**−132**	66	48	**−83**	197	340	**−30**
93TH975	52	**−259**	**−72**	**−3**	307	430	418	**−228**	81	66	**−141**	90	423	**−89**
92TH024	**−176**	**−70**	**−250**	15	158	56	150	**−36**	55	156	**−315**	191	343	**−27**

[a] Values were calculated from neutralization data presented in the accompanying report (39). Values derived from autologous neutralizations are enclosed by boxes; negative values are in boldface. HIV-1 isolates and sera were obtained from NIAID and WHO.

[b] ND, phenotype not determined.

likely to significantly dictate the nature of the neutralization serotypes. Furthermore, serum-dependent infectivity enhancement phenomena were commonly observed in our studies and seem to be isolate rather than serum specific; SI isolates were slightly more likely than NSI isolates to be susceptible to infectivity enhancement. Together, these findings have strategic implications for the development of any globally effective HIV-1 vaccine. Although a more serious obstacle, understanding how to induce by vaccination broadly neutralizing antibodies in the absence of infection-enhancing antibodies, remains, a more comprehensive analysis of serum neutralization of HIV-1 is necessary to make definitive decisions about the exact number and nature of all neutralization serotypes of HIV-1.

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