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Neutralization Serotypes of Human Immunodeficiency Virus Type 1 Field Isolates Are Not Predicted by Genetic Subtype

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Human immunodeficiency virus type 1 (HIV-1) primary isolates from four geographical locations in Thailand, Brazil, Rwanda, and Uganda, representing genetic subtypes A, B, C, D, and E, were examined for autologous and heterologous neutralization by panels of human HIV$^+$ polyclonal plasma. In independent linked experiments in three laboratories using diverse methodologies and common reagents, no defined pattern of genetic subtype-specific neutralization was observed. Most plasma tested were broadly cross-neutralizing across two or more genetic subtypes, although the titer of neutralization varied across a wide range. We conclude that the genetic subtypes of HIV-1 are not classical neutralization serotypes.

Human immunodeficiency virus type 1 (HIV-1) is characterized by remarkable genetic diversity (6, 12), which has been classified phylogenetically into one main group of HIV-1 isolates (group M) and one outlier group (group O) (18). The main group currently consists of eight genetic subtypes, A to H (21). Typically, intrasubtype nucleotide sequences vary by 5 to 15% and intersubtype sequences vary by up to 30% (21). We have addressed the biological significance of these genetic subtypes in relation to vaccine development. The Global Programme on AIDS of the World Health Organization (WHO) has collaborated in the development of potential field sites for future phase III trials of HIV/AIDS vaccines in Rwanda, Uganda, Thailand, and Brazil (26). To better define the HIV-1 strains circulating in these countries, an international network of laboratories undertook the task of isolating and extensively characterizing the HIV-1 strains in each site. At least 30 HIV-1 isolates from each site have been genetically subtyped by the heteroduplex mobility assay (7), and representative strains have been sequenced in the V3, C2/V3, and/or complete gp120 or gp160 region (10). In this survey, Rwandan isolates were all genetic subtype A; Ugandan isolates were genetic subtype A or D; and Brazilian isolates were predominantly genetic subtype B, including a B′ variant, with rare C and F subtypes identified. The Thailand isolates were predominantly genetic subtype E, with a Thai B variant identified in a small minority (26). According to phylogenetic classification, all of these isolates belong to the main group of HIV-1 isolates (18, 21).

To determine whether these genetic subtypes based on phylogenetic analyses of nucleotide sequences also define functional neutralization types (classical serotypes), the immunotyping group of the WHO Network examined the neutralization of representative HIV-1 field isolates from genetic subtypes A to E. Type-specific antisera raised to recombinant Env proteins do not neutralize field isolates passaged in peripheral blood mononuclear cells (PBMCs) (4, 20); however, field isolates can be neutralized by polyclonal sera from HIV$^+$ subjects (1, 3). Sera from HIV$^+$ subjects infected with diverse genetic subtypes exhibit type specificity in terms of Env peptide binding (5). Thus, the neutralization titers of autologous and heterologous plasma samples were assayed in a checkerboard fashion, using the WHO panel of field isolates from genetic subtypes A to E; the plasma samples were mostly obtained from subjects who had seroconverted within 2 years prior to blood collection. Initial studies were carried out with pooled plasma from each geographical location, and subsequent checkerboard analyses were undertaken with individual plasma samples. Identical aliquots of well-characterized viral stocks of primary field isolates, passaged one to two times through donor PBMCs, were used in all assays. Common reagents were used in three laboratories to assess the reproducibility of each assay format and hence the validity of testing in separate laboratories.

MATERIALS AND METHODS

Each of the three participating laboratories (Imperial College, London, United Kingdom [IC], Karolinska Institute, Stockholm, Sweden [KI], and National Cancer Institute, Bethesda, Md. [NCI]) used their own techniques and controls (Table 1); a common positive control plasma (FDA#2) was used in each laboratory, and a panel of common plasma and viruses was studied to compensate for interlaboratory variation. Positive controls from each participating laboratory were selected from HIV$^+$ subjects previously shown to have high-titer neutralizing antibodies against subtype B field isolates in PBMCs. All assays used 3-day phytohemagglutinin (PHA)-stimulated donor PBMCs, with viral production measured by p24 antigen (p24Ag) enzyme-linked immunosorbent assay (ELISA). In two laboratories (IC and KI), neutralization assays were carried out with serial twofold dilutions of heat-inactivated plasma in multiple replicates, with a fixed quantity of input virus (50 tissue culture infective doses for IC and 15 for KI). Neutralization titers were defined after 7 days of incubation as the reciprocal of the highest dilution of plasma giving 50% (IC) or 90% (KI) reduction in p24Ag compared with an HIV-negative control plasma. The third laboratory (NCI) used an infectivity reduction format, with a fixed dilution of plasma (final dilution, 1:20) and fivefold dilutions of viral stock. Neutralization titer in

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TABLE 1. Comparison of neutralization assays in the three laboratories[a]

Laboratory	PBMC source[b]	No. of PBMC/well	Plasma dilution	Lower titer limit	Input virus	Endpoint reduction
IC	Single	10^5	1:10–1:320	<10	50 ID_{50}	50% p24Ag reduction
KI	Double	10^5	1:20–1:320	<20	15 ID_{50}	90% p24Ag reduction
NCI	Double	2×10^5	1:20	<10	5-fold dilutions	10-fold virus titer reduction

[a] Parameters of input virus, cells, and plasma are shown for each of the three laboratories. In all cases, 96-well plates were used, the endpoint was 7 days, and p24 was measured.

[b] Refers to number of donors used following cell separation and activation.

this assay was determined by the $\log_{10}$ reduction of viral infectivity by the test plasma compared with an HIV^- plasma control. A 10-fold reduction of infectivity was considered neutralization. A number of washing techniques were adopted to abrogate interference from input anti-p24 antibody on the p24Ag ELISA readout (2); the IC laboratory also used an anti-p24 antibody from a Ty.p24.VLP vaccine study (24) in which the anti-p24 antibody titer is in a range similar to that found in clinical samples.

Neutralization assays and ID_{50} determinations carried out at IC and KI. (i) Primary virus titration. In the IC laboratory, virus isolates were titrated in five replicates from 1:5 to 1:15,625 with 10^5 3-day PHA (5 μg/ml; Sigma)-stimulated donor PBMCs from a single donor, in RPMI 1640 (Gibco) with 15% fetal calf serum (FCS; Seralab), interleukin-2 (IL-2; 10 IU/ml; MRC-ADP), and antibiotics, in 96-well round-bottom plates (Corning). The supernatant was changed on days 1 and 3, and 100 μl of supernatant was taken on day 7 for HIV-1 p24Ag determination by in-house ELISA (15). The viral 50% infective dose (ID_{50}) was calculated by the Reed-Muench formula.

The KI laboratory used a virus titration technique (2) similar to that described above except that (i) PBMCs from two different donors were stimulated with 2.5 μg of PHA (Difco) per ml and (ii) medium containing 10% FCS (Gibco), 5 IU of recombinant IL-2 (Amersham) per ml, and 2 μg of Polybrene (Sigma) per ml was used throughout. The in-house ELISA is described in reference 23.

(ii) Neutralization assay. In the IC laboratory, the plasma was heat inactivated at 56°C for 30 min and serially diluted in duplicate from 1:5 to 1:160 in a 50-μl volume. Virus was added at a constant 50 ID_{50}, the plates were incubated for 1 h at 37°C, and then 10^5 PHA-stimulated PBMCs were added, to make a final volume of 200 μl. The plates were washed on days 1 and 3, and the supernatant was taken at day 7 for p24Ag assay. The controls used were wells with cells only, virus only, and cells and virus with no plasma. The neutralizing titer of a particular plasma and virus was defined as the reciprocal of the highest dilution giving a 50% reduction in p24Ag by ELISA compared with the HIV^- plasma control.

Neutralization assays in the KI laboratory were run simultaneously with ID_{50} titrations, using three virus dilutions for each plasma sample (2). The plasma were diluted in triplicate directly in the plate, in a total volume of 75 μl. Five steps of twofold dilutions, starting with 1:10, were used. Virus was then added in an equal volume, and the test carried out as at IC. The negative virus controls consisted of five wells with virus but no cells and five wells with cells but no virus. A serum from an asymptomatic Swedish subject selected for high-titer neutralizing antibodies (SE1785) was used as positive serum control. Neutralization was evaluated with 15 infective doses. The neutralizing titer of a plasma was defined as the reciprocal of the highest dilution giving a 90% reduction in absorbance value in the p24Ag assay.

Infectivity reduction assay (NCI). The same pool of normal donor PBMCs was used for each panel of plasma. All plasmas were heat inactivated at 56°C for 30 min and then centrifuged at 4,000 rpm for 15 min. FDA#2 plasma was obtained through Ogden Bioservices Inc., and normal human serum was from the same pooled serum lot (Gibco, Grand Island, N.Y.). For each assay, a mock-treated virus only (no cells) control was included to establish that residual p24 was effectively diluted to remove background p24. PHA-stimulated PBMCs were treated with Polybrene and prepared as described above. Wash medium (RPMI 1640, 15% FCS, 1% L-glutamine, 1% penicillin/streptomycin) was added to the A and H rows of a V-bottom microtiter culture plate to minimize evaporation loss from inner wells. PBMCs were dispensed at 20 μl (2×10^5) to the appropriate wells of the culture plate. Heat-inactivated geographic plasmas were diluted at threefold the desired final concentration of 1:20 with heat-inactivated normal human serum, and 20 μl of the diluted plasma was added to five replicate wells for each virus dilution. Virus titrations were performed at four- or fivefold dilutions, depending on the range of dilutions necessary to achieve an endpoint with all test samples. Viral dilutions were prepared in a separate plate at three times the final desired dilution, and then a 20-μl volume of the diluted virus (five wells per dilution) was added to the cells and plasma for a final volume of 60 μl in the infectivity assay. The virus-alone control wells contained 40 μl of coculture medium (wash medium plus 32 IU of IL-2 per ml) instead of PBMCs and antibody. The plate was placed in an incubator, and cells were cultured overnight (16 to 18) at 37°C, 5% CO_2, and 95% humidity. The next morning, 2.0 ml of wash medium was added to each corresponding well of a 96-well Cube 2ube (DBM, Valencia, Calif.) for each culture plate to be washed. The 60-μl volume of cells and supernatant from V-bottom culture plates was gently resuspended and transferred to the Cube 2ube. Following the transfer, each well of the V-bottom culture plate was rinsed with 200 μl of wash medium taken from the Cube 2ube wells. The Cube 2ube plates were then centrifuged for 15 min at $220 \times g$, and 1.9 ml of wash medium was removed by aspiration. This step was performed twice more. After the third and final wash, the cells were gently resuspended in the remaining 100 μl and transferred from the Cube 2ube to an appropriately labeled 96-well U-bottom microtiter culture plate. Each well of the Cube 2ube was rinsed with 100 μl of coculture medium containing twice the normal concentration of IL-2, the rinse was added to the appropriate wells of the microtiter plate to create a final volume of 200 μl, and the plates were placed in a 5% CO_2 incubator. On day 4, the cells and supernatant were gently resuspended in all wells, a 125-μl sample was removed, and the original culture plate was refed with 150 μl of fresh coculture medium. The day 4 supernatant was saved in a 96-well plate for p24Ag capture analysis. At day 7, the original culture plate was centrifuged for 15 min at $220 \times g$ and 150 μl of supernatant was transferred to a 96-well plate for p24Ag capture analysis (Dupont ELISA kit).

Calculation of neutralization index or relative difference was performed as follows. For each virus isolate used in infectivity reduction assays, a parallel titration was performed in the presence of NHS alone as a reference for virus growth. Each well in a viral dilution assay was scored plus or minus by comparing its p24 count with a cutoff. The cutoff used was mean background plus 3 standard deviations. ID_{50}s and associated errors were then calculated by one of three methods, the model fit, the curve fit, or Spearman-Karber, with the computer program ID-50, which can run on Macintosh or IBM computers under Windows 2.1, as previously described (22).

RESULTS

A comparison of the input virus concentration, number of PBMCs, and titration of plasma among the laboratories is shown in Table 1.

Pooled plasma neutralization (IC). Initially, pools of plasma samples from each geographical location, representing Rwanda (A), Brazil (B), Thailand (E/B), and Uganda (A/D), with an additional subtype B plasma pool from UK HIV^+ subjects, were made. Neutralization was studied in the IC lab-

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TABLE 2. Pooled plasma neutralization titers[a]

Subtype	Pooled plasma origin	Endpoint neutralization titer for indicated WHO primary virus isolate and genetic subtype									
		A			B				D		
		UG029	UG031	RW009	BR014	BR020	BR021	TH014	UG024	UG038	UG046
A/D	Uganda	20	40	20	20	80	20	80	10	10	10
A	Rwanda	80	80	40	40	80	40	80	20	20	40
B	Brazil	10	10	10	10	20	10	10	—	—	—
E/B	Thailand	—	20	10	10	20	10	10	—	—	10
B	United Kingdom	20	80	40	80	>320	80	>320	20	20	40

[a] Endpoint titers (50% p24Ag reduction), from the IC laboratory, of plasma pools from the four sites against 10 field isolates from genetic subtypes A, B, and D, with a U.K. plasma pool from HIV^+ homosexual men ($n = 5$) infected with the B subtype as a control. —, neutralization titer of <10.

oratory as outlined in Table 1. No type specificity of neutralization was demonstrated by these pools on 10 virus isolates from genetic subtypes A, B, and D, while plasma pooled from the UK produced the highest titers of cross-neutralization (Table 2). This finding led us to conclude that neutralization assays would have to be performed with individual plasma samples.

Autologous and heterologous neutralization. Checkerboard panels of neutralization of autologous and heterologous viruses and plasmas were then undertaken in the two participating laboratories (Tables 3 and 4). In Table 3 (IC), which shows neutralization titers of seven plasma samples against five field isolates from genetic subtypes A to D, high-titer autologous neutralization (1:80) is seen only for one plasma-virus combination (RW009). Autologous neutralization was weak (≤1:20) or absent in four of five pairs. The broadest cross-neutralization is observed with the FDA#2 plasma and with the subtype B U.K. control plasma M36321 (from a London patient with AIDS), which neutralized four of five and five of five field isolates, respectively, irrespective of genetic subtype. The control anti-p24 plasma was consistently negative, which illustrates the effectiveness of the washing techniques. Table 4 (KI) shows the neutralization titers of 12 plasma samples against eight field isolates representing genetic subtypes A to E. Autologous neutralization is weak or absent, with only UG037, RW009, and UG038 showing high (1:80) titers. No genetic subtype-specific pattern is discernible from these data. Instead, broadly cross-neutralizing activity was demonstrated in five cases. SE1785 (the Swedish positive control serum; presumed subtype B), RW008, and TH022 neutralized seven of eight viruses tested, spanning all five subtypes. Neutralization titers were generally low (1:20 to 1:40) with the TH022 plasma and high with SE1785 (1:80 to >1:320), two to eight times higher than with RW008. Thus, SE1785 was the broadest and most potent cross-neutralizing reagent in this panel. Next-best plasma samples were TH026 and FDA#2, both neutralizing six of eight viruses. TH026 failed to neutralize subtype C, while FDA#2 failed to neutralize subtype D.

The IC and KI laboratories used their own neutralization assay methodologies; however, assays based on the growth of primary viruses in donor PBMCs are subject to biological variation, of the order of 0.6 $\log_{10}$, which has been found repeatedly in studies requiring the quantitative cocultivation of HIV-1 (9, 17). Interassay variation within the IC laboratory ranged over ±1 dilution on seven replicates of a common primary virus and plasma pair (data not shown). Neutralization titers on virus stocks and plasma samples common to both laboratories and a third collaborating laboratory (NCI) are shown in Fig. 1. Figures 1A to E show representative plasma from subjects infected by genetic subtypes A to E; titers against the five field isolates representing genetic subtypes A, B, C, and D are generally low; laboratories are consistent within ±1 dilution for 60 of 66 assays (91%). In Fig. 1F, the titers of the FDA#2 plasma against the five field isolates show a range of neutralization over approximately 0.6 $\log_{10}$ for any plasma-virus combination; each laboratory scored a different virus highest. Only one virus (UG024) was not neutralized by FDA#2, and this finding was consistent in each laboratory. These data demonstrate that the titers of neutralization are comparable to within 0.6 $\log_{10}$, irrespective of assay methodology, and that high-titer and low-titer plasma samples are recognized comparably by all laboratories. This variation is similar to that seen in the comparative neutralization study by D'Souza et al. (8).

DISCUSSION

These data, from three laboratories with independent but comparable assays and common reagents, demonstrate that the pattern of neutralization of HIV-1 field isolates is not simply related to genetic subtype. The checkerboards undertaken at IC and KI show no clear pattern of neutralization in relation to genetic subtype. While it is possible to identify monospecific plasma, the cross-reactivity of primary virus neu-

TABLE 3. Autologous and heterologous neutralization titers (IC)[a]

Subtype	Plasma	Endpoint neutralization titer for indicated WHO primary virus isolate and genetic subtype				
		A, RW009	B		C, BR025	D, UG024
			BR020	TH014		
A	UG037	>160	—	40	20	—
	RW009	**80**	—	10	—	—
B	BR020	10	**—**	10	—	—
	TH014	10	—	**20**	—	—
C	BR025	80	—	20	**10**	10
D	UG024	20	—	10	—	**—**
E	TH022	80	—	40	—	—
	FDA#2	80	40	>160	40	—
	M36321	>160	40	>160	20	40
	Tp.24.VLP αp24	—	—	—	—	—

[a] Full checkerboard of autologous and heterologous endpoint neutralization titers (50% p24Ag reduction) of seven plasmas from subjects infected by genetic subtypes A to E against five viruses isolated from the same subjects. —, titer of <10. The boxed figures show autologous neutralization. FDA#2 was a common positive control pooled plasma first produced by the U.S. Food and Drug Administration, derived on three occasions from a single HIV^+ homosexual male from the United States, and presumed to be subtype B. Plasma M36321 was a positive control plasma from a U.K. HIV^+ homosexual male with AIDS, and the Ty.p24.VLP plasma was from a vaccine study with a p24Ag in healthy HIV^- volunteers (14). The methodology was identical to that used for Table 2.

TABLE 4. Autologous and heterologous neutralization titer (KI)[a]

Subtype	Plasma	Endpoint neutralization titer for indicated WHO primary virus isolate and genetic subtype							
		A		B		C, BR025	D		E, TH/BKK10
		UG037	RW009	BR020	TH014		UG024	UG038	
A	UG037	[80]	40	20	—	40	—	—	—
	RW008	40	80	80	—	40	20	160	40
	RW009	—	[80]	—	—	ND	—	—	—
B	BR003	—	40	20	80	—	—	20	320
	BR020	80	20	[—]	—	—	—	—	—
	TH014	ND	20	—	[—]	ND	—	—	ND
	TH026	40	40	20	20	—	20	—	320
C	BR025	20	—	—	—	ND	—	20	—
D	UG024	—	40	20	—	—	[20]	20	40
	UG038	20	40	—	80	—	80	[80]	—
E	TH011	40	—	—	—	—	—	—	20
	TH022	40	20	40	—	20	20	20	20
	FDA#2	80	40	80	160	>320	—	—	160
	SE1785	160	160	160	80	>320	80	—	320

[a] Full checkerboard of autologous and heterologous endpoint neutralization titers (90% p24Ag reduction) of 12 plasma samples against eight viruses, partly from the same subjects. —, titer of <10; ND, not done. Autologous neutralization is shown by the boxed figures. FDA#2 is a positive control plasma (see Table 3); SE1785 is a positive control serum from a Swedish HIV$^+$ homosexual male, presumed to be subtype B, selected because of prior evidence of broad neutralizing activity against primary isolates.

tralization across genetic subtypes is the dominant interpretation of these data.

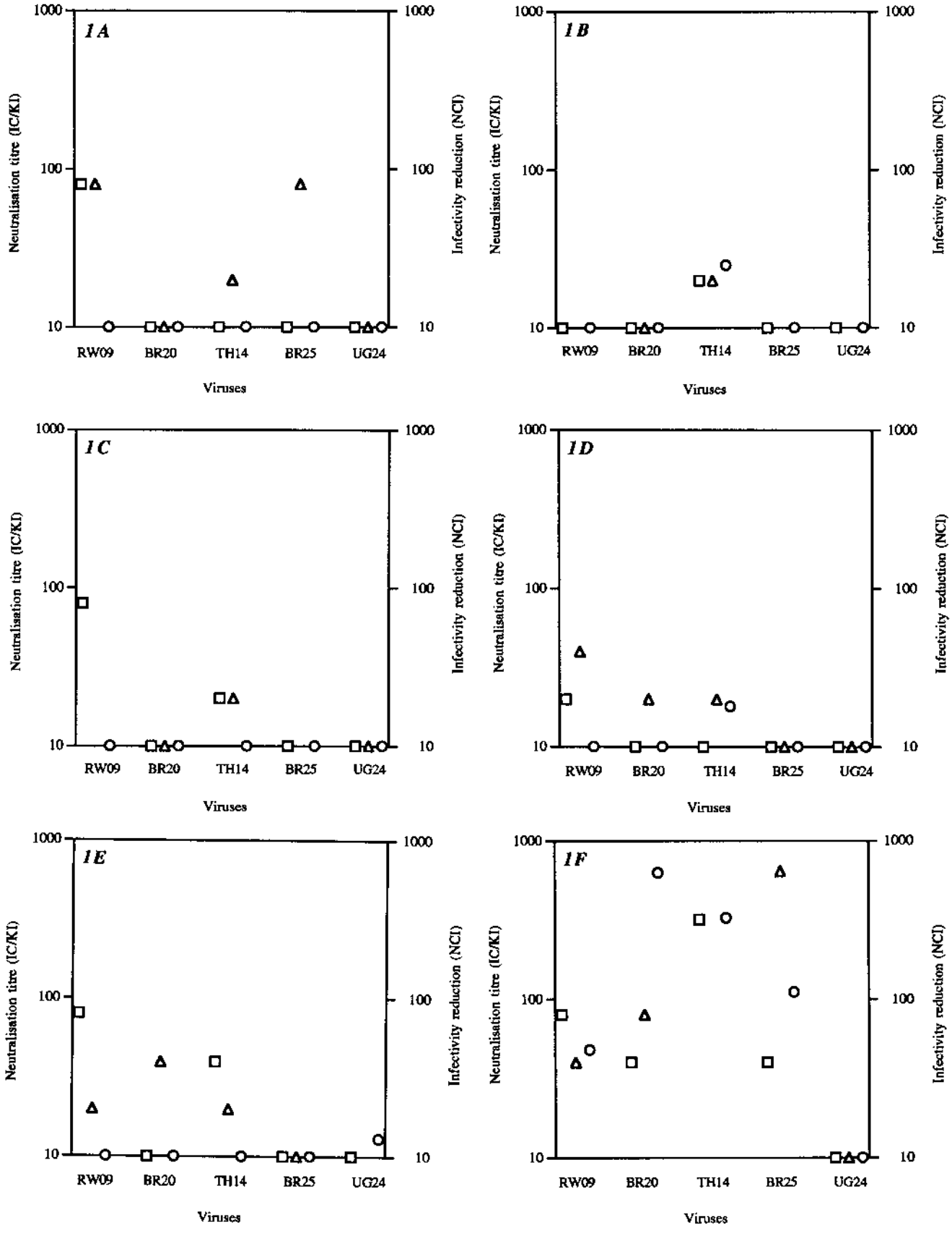
These results are entirely consistent with the data published by Weiss et al. in 1985 (25), from a study using laboratory-adapted viruses in immortalized T-cell lines, which demonstrated that sera from U.K. and Ugandan HIV-1$^+$ subjects were equally capable of neutralizing North American or African HIV-1 isolates. In that study, sera either failed to neutralize or exhibited broad neutralization across all viruses tested. Similarly, viruses either were relatively resistant to neutralization or were neutralized equally by all potent plasmas. In the present study also, plasma samples showed differences in the capacity to neutralize. One-third of the plasma samples can be said to be broadly cross-neutralizing, with the selected positive control plasmas M36321 (IC), FDA#2 (tested by both laboratories), and SE1785 (KI) found to be the most potent. The rest of the plasmas show a spectrum of neutralizing activities, with BR020 plasma being the weakest. With respect to the viruses, the RW009 isolate appeared to be the most sensitive to neutralization and was neutralized by all nine plasmas tested at IC and by 12 of 14 plasma tested at KI. In contrast, the UG024 isolate showed relative resistance to neutralization and was neutralized by 2 of 9 (IC) and 6 of 14 (KI) plasmas. Like plasmas, viruses showed a spectrum of sensitivities rather than falling into clear-cut groups. This difference from the study by Weiss et al. (25) may be explained by the larger amount of material tested and by the fact that primary isolates were tested on PBMCs.

More recently, Mascola et al. have reported that the B and E subtypes in Thailand may be discrete neutralization serotypes (13). Sera from subjects infected by B and E genetic subtypes are the most discrete and diverse in V3 peptide binding assays (5, 19); however, subtype E plasmas analyzed in our studies were generally weak neutralizers of all viruses, while potent subtype B plasmas reproducibly neutralized subtype E virus. Our results are in line with those reported by Kostrikis et al. (11) and Moore et al. (16); i.e., no neutralization pattern consistent with genetic subtypes can be documented, even when a large number of sera and isolates are tested. With respect to the V3 peptide binding data, subtype B and E viruses represent the far poles of an antigenic spectrum, making the weak cross-neutralization seen in Table 4 even more significant.

The *env* sequence-derived genetic subtypes can be represented by peptides representing V3 consensus sequences; sera from HIV$^+$ subjects bind to these V3 peptides representing subtypes A to E in a type-specific manner (5). As binding can be shown to be at least in part subtype specific, the presence of cross-neutralization rather than type-specific neutralization suggests that the V3 epitope does not contribute significantly to the capacity of human HIV$^+$ sera or plasma to neutralize field isolates of HIV-1 in PBMCs. Interestingly, another study using chimeric viruses carrying the gp120 portion of the envelope from primary HIV-1 isolates on the HXB2 backbone led to a similar conclusion (14). The cross-neutralizing activity in human sera appeared to be directed to epitopes outside gp120. This observation remains to be confirmed by further study but may be highly significant to future vaccine development.

In accordance with other studies (11, 16), we may conclude that the genetic subtypes based on the phylogeny of *env* sequences are not classical neutralization serotypes. The implications of these findings for vaccine research are both encouraging and frustrating. First, even at the level of first-passage field isolates of HIV-1 from potential phase III vaccine field

FIG. 1. Interlaboratory comparison of neutralization. The neutralization titers of six plasma samples from subjects infected with genetic subtypes A to E were compared among the three laboratories, using five common first- or second-passage field isolates from genetic subtypes A, B, C, and D. The laboratories (□, IC; △, KI; ○, NCI) used independent methodologies as outlined in Table 1. Shown are neutralization titers of virus isolates RW009 (subtype A), BR020 (subtype B), TH014 (subtype B), BR025 (subtype C), and UG024 (subtype D) against plasmas RW009 (subtype A) (A), TH014 (subtype B) (B), BR025 (subtype C) (C), UG024 (subtype D) (D), TH022 (subtype E) (E), and FDA#2 (F). Endpoint neutralization titers in the IC and KI assays (Tables 3 and 4) are shown on the left-hand *y* axis; the titers in the infectivity reduction assay (NCI) are shown on the right-hand *y* axis (methodology in Table 1). The cutoff for the IC data is <10, and that for the KI data is <20 (Table 1); for the purposes of these comparisons, negative values are taken as ≤10.


1A
1B
1C
1D
1E
1F
Neutralisation titre (IC/KI)
Infectivity reduction (NCI)
1000
100
10
RW09
BR20
TH14
BR25
UG24
Viruses

sites, cross-neutralizing epitopes dominate in HIV^+ human sera and plasmas. However, to date antibodies to these epitopes have not been induced by immunization with recombinant proteins. If an immune response to these epitopes is important in conferring protection against HIV and can be elicited by immunization, the considerable genetic variation of HIV-1 may not preclude a successful HIV vaccine.

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