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Standard Conditions of Virus Isolation Reveal Biological Variability of HIV Type 1 in Different Regions of the World

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ABSTRACT

HIV-1 isolates were obtained from four countries within the framework of the WHO Network for HIV Isolation and Characterization. The use of standard HIV isolation procedures allowed us to compare the biological properties of 126 HIV-1 isolates spanning five genetic subtypes. In primary isolation cultures, viruses from Uganda and Brazil appeared early and replicated without delay, whereas the replication of Thai viruses was delayed by several weeks. Regardless of genetic subtype or country of origin, blood samples collected more than 2 years after seroconversion yielded virus that replicated efficiently in the primary isolation cultures. None of the isolates obtained from Thailand or Rwanda replicated in cell lines, whereas 5 of the 13 Brazilian isolates and 7 of the 11 Ugandan isolates replicated and induced syncytia in MT-2 cells. As expected for virus isolates obtained early in HIV-1 infection (within 2 years of seroconversion), all viruses from Brazil, Rwanda, and Thailand showed a slow/low replicative pattern. For the Ugandan samples, the time from seroconversion was known precisely for a few of the samples and only in one case was less than 2 years. This may explain why the five viruses that were able to replicate in all cell lines, and thus classified as rapid/high, were of Ugandan origin. Viruses able to induce syncytia in MT-2 cells, also induced syncytia in PBMC. However, 8 slow/low viruses (out of 27) gave discordant results, inducing syncytia in PBMC but not in MT-2 cells. Furthermore, using syncytium induction as a marker, changes in virus populations during early *in vitro* passage in PBMC could be observed. The results indicate that biological variation is a general property of HIV-1 in different regions of the world. Moreover, the time from HIV-1 infection, rather than genetic subtype, seems to be linked to viral phenotype.

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INTRODUCTION

HUMAN IMMUNODEFICIENCY VIRUS (HIV) isolates have been shown to differ in their replicative and cytopathic characteristics.[1] With genetic subtype B viruses from Europe and North America, the characteristics of isolated viruses have been shown to vary in relation to the severity of HIV infection.[2–6] Notably, individuals with asymptomatic HIV infection yielded, as a rule, virus that replicated slowly and to low titers in peripheral blood mononuclear cell (PBMC) cultures, did not replicate in cell lines and lacked syncytia-inducing capacity. In contrast, patients with immunodeficiency yielded virus able to efficiently replicate not only in PBMC but also in cell lines, and which induced large syncytia. Accordingly, HIV isolates have been classified as slow/low or non-syncytia-inducing and rapid/high or syncytia-inducing.[2,4,6,8] Slow/low viruses could be further subdivided according to infectivity titers in PBMC, numbers of viral RNA molecules in infected cells, replication in monocyte/macrophage cultures, and their ability to transiently replicate in cell lines.[3,7–9] When patients with progressive HIV-1 infection were followed over time, a gradual increase in the replicative capacity of isolated viruses could be demonstrated *in vitro*.[9–11] This led to the suggestion that the increased capacity to replicate *in vitro* signals the appearance of a more virulent virus.[10,11] Indeed, in some cases, the emergence of virus able to replicate in cell lines and with syncytia-inducing capacity immediately precedes a sharp decline in CD4 counts. However, this is not always the case and CD4 counts may decrease and the patient may develop AIDS without the emergence of such viruses.[8,12,13]

The present study, performed within the framework of the WHO Network for HIV Isolation and Characterization, provided the opportunity to address the question of whether HIV-1 isolates of genetic subtypes other than B differ in biological properties. HIV-1 isolates were carefully observed for changes in biological properties in primary culture and during subsequent passage in PBMC. Also, this collaborative study made it possible to systematically compare different classification systems for HIV-1 biological phenotyping and to relate phenotypic differences to V3 loop sequence.

MATERIALS AND METHODS

HIV-1 isolation

Blood was collected from HIV-1-infected asymptomatic individuals in Brazil, Rwanda, Thailand, and Uganda as described[14] and shipped to the centralized HIV isolation laboratories, at the Georg-Speyer-House (GSH) in Frankfurt and at the National Institute for Biological Standards and Control (NIBSC) in London. All shipments were received within the time indicated in Table 1. Blood was drawn in identical syringes (Sarstedt). For virus isolation, a standardized protocol based on the coculturing of patient's peripheral blood mononuclear cells (PBMC) with donor PBMC was used.[14] Donor PBMC were derived by separation of buffy coats on Ficoll gradient (NIBSC: Pharmacia, GSH: Biochrom) and stimulated for 3 days with 5–10 μg/ml phytohemagglutinin (PHA-P, Sigma) in RPMI 1640 medium containing 15% fetal calf serum (FCS, NIBSC: Gibco, GSH: Boehringer-Mannheim) and antibiotics. Donor PBMC (12×10^6) were then mixed with at least 5×10^6 PBMC from the infected subjects (similarly separated on Ficoll gradient) and cultured in 14 ml medium containing 10–20 U/ml recombinant interleukin 2 (rIL-2, NIBSC: MRC AIDS Reagent Project, GSH: Boeringer-Mannheim). Cultures were monitored

TABLE 1. HIV-1 ISOLATION EFFICIENCIES FROM BLOOD SAMPLES OBTAINED FROM FOUR WHO NETWORK PRIMARY LABORATORIES[a]

Country	*Time in transit (days)*	*Number of blood samples*	*Positive isolations*	
			Number	*Percent*
Brazil[b]	2	43	37	86
Rwanda	2	19	13	68
	3	7	6	
		Total: 26	19	73
Thailand	≤2	69	31	45
	4	11	0	0
		Total: 80	31	39
Uganda	≤2	69	27	39
	3	20	6	30
	≥4	9	3	
		Total: 98	36	37

[a]Total of 241 samples as of March 1994. Isolation frequencies are higher for Brazil, Rwanda, and Uganda as compared to the material collected until September 1993.[14]

[b]In addition, nine samples were contaminated, and were not included in the calculation.

for viral growth by p24 antigen assay (NIBSC: Coulter, GSH: Innogenetics) twice a week for at least 4 weeks. The cultures were also tested for reverse transcriptase activity[6] and inspected microscopically for cytopathic effects at regular intervals.

Infection of donor PBMC

Frozen supernatants from virus isolation cultures were shipped to the Karolinska Institute (KI) in Stockholm, where further biological characterization was carried out. Separation of HIV-negative donor PBMC and culture conditions were similar to those described above, with some differences as follows. The PHA concentration used for stimulation of donor PBMC was 2.5 μg/ml and the RPMI medium was supplemented with 10% FCS (Flow), 2 μg/ml polybrene (Sigma), and 10 U/ml rIL-2 (Amersham). PBMC cultures were infected with 1 ml of early virus-positive supernatant from primary isolation cultures, observed for cytopathic effects, and culture supernatants tested twice a week for reverse transcriptase activity and p24 antigen.[15,16]

Determination of replication pattern

HIV-1 isolates were passaged twice in PHA-stimulated donor PBMC before the assay for replicative capacity could be carried out. In the assay, PBMC were infected with 20,000 cpm/10^6 cells reverse transcriptase (RT) activity. Infected PBMC were cocultivated with cell lines at day 7 or 10, at the time of peak RT activity. For cocultivation 1×10^6 PBMC were mixed with 3×10^6 of each of the cell lines, U937-2, CEM, MT-2, and Jurkat-tat, as described.[3] MT-2 cell cultures were tested for p24 antigen and observed for syncytia. Virus growth was considered to be positive when virus was detected in at least three consecutive tests.

RESULTS

Replicative capacity of HIV-1 isolates in primary isolation cultures

HIV-1 isolation efficiencies varied widely between the four sites (Table 1). Considering those samples that spent a maximum of 2 days in transit, the lowest isolation frequency (39%) was obtained with the Ugandan samples and the highest (86%) with the Brazilian samples. While 4 days in transit severely impaired the ability to isolate HIV-1 from Thai samples (0%), it had only a minimal effect on the isolation frequency of Ugandan samples; after 4 days in transit one-third of samples still yielded virus. The results suggest that a particular type of virus that readily replicates in *in vitro* culture is recovered from Ugandan samples, albeit from a minority, by the isolation procedure adapted. Another parameter that could conceivably affect isolation frequency is the number of patient's cells used in the primary isolation culture. In the present experiments a minimum of 5×10^6 cells was used from each infected individual. Under such conditions the cell numbers did not influence isolation efficiency (data not shown).

To characterize the replicative capacity of viruses in primary isolation cultures, two parameters were used. One is the time necessary to obtain the first p24 antigen-positive culture and the second is the time necessary to reach an OD value of 1. In parallel with antigen determinations, reverse transcriptase assays were also performed at GSH, with similar results (data not shown). While appearance of detectable virus in the primary isolation culture may depend on the patient's viral load at the time of sample collection, the time necessary for abundant virus replication (OD = 1) is an indicator of virus replicative capacity. To eliminate differences due to time after seroconversion, the replicative capacity of viruses has been depicted as a func-

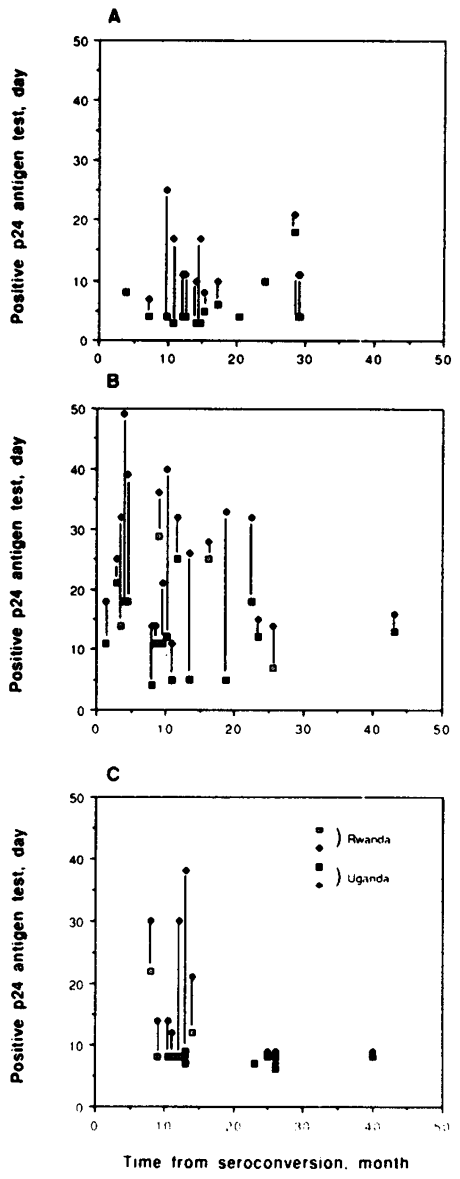

FIG. 1. Virus replication in primary isolation cultures as a function of time after seroconversion. Each isolate is characterized by the number of days to first HIV-1 p24 antigen-positive culture supernatant (square) and to antigen value OD = 1 (diamond). These two are coincidental whenever the first antigen-positive value is higher than OD 1. HIV-1 isolates were obtained from Brazil (A), Thailand (B), Rwanda (C), and Uganda (C).

TABLE 2. REPLICATIVE CHARACTERISTICS OF HIV-1 ISOLATES IN CELL LINES

Country	Genetic subtype HMA[b]	Replicative pattern[a] PBMC	s/l 1	s/l 2	s/l 3	r/h
Brazil	B	13		8	5	
	C	1		1		
Rwanda	A	6	1	5		
Thailand	B	2	2			
	E	12	1	11		
Uganda	A	3		2	1	
	D	8		2	1	5

[a]r/h, rapid/high; s/l, slow/low; s/l 1, replication in PBMC only; s/l 2, replication in PBMC and Jurkat-tat cells but not in other cell lines; s/l 3, in addition to replication in PBMC and Jurkat-tat cells, replication and syncytium induction in MT-2 cells and/or transient replication in other cell line(s); r/h, replication on at least one cell line in addition to MT-2 and syncytium induction (modified after Fenyö *et al.*[3]).

[b]HMA, heteroduplex mobility assay, makes use of a 1.2-kb envelope fragment generated by nested PCR.[17]

tion of time from seroconversion (Fig. 1). HIV-1 was detected in cultures from Brazilian samples within a week and an OD of 1 was reached in most cases within the second week of culture (Fig. 1A). In contrast, the majority of Thai samples yielded an appreciable amount of virus (OD = 1) only during the third week of culturing or later, even when virus was detectable at an early time (Fig. 1B). Viruses isolated from Rwandan samples showed an intermediary replication pattern (Fig. 1C) between the rapid Brazil and slower Thai viruses. Interestingly, in all three groups virus isolates derived from subjects with HIV-1 infection over 2 years tended to appear early and/or to replicate in culture with minimal delay.

Replicative capacity of HIV-1 isolates in cell lines

Virus isolates were classified according to their capacity for growth in PBMC and in the Jurkat-tat, MT-2, CEM, and U937-2 cell lines. Viruses replicating on PBMC but not in any of the cell lines were rated as slow/low group 1 (s/l 1); if they also replicated on Jurkat-tat cells, they were rated s/l 2; if they, in addition to replication on PBMC and Jurkat-tat cells, replicated and induced syncytia in MT-2 cells and/or replicated transiently in other cell line(s), they were rated s/l 3. Rapid/high (r/h) designates replication on at least one cell line in addition to MT-2 and syncytium-inducing capacity (modified from Fenyö *et al.*[3] A summary of viral isolate growth in relation to country of origin and genetic subtype is given in Table 2. Genetic screening was performed by use of the heteroduplex mobility assay on a 1.2-kb envelope fragment generated by nested PCR.[17]

Five of the 13 Brazilian isolates of genetic subtype B, but none of the isolates from Thailand (subtype B or E) or Rwanda (subtype A), were able to replicate and induce syncytia in MT-2 cells. This is in line with the differences observed in primary cultures between HIV-1 isolates from these sites. Seven of the 11 Ugandan isolates readily replicated in at least one cell line.[14]

Syncytium-inducing capacity of HIV-1 isolates in primary cultures and during early passage in PBMC

Two Brazilian viruses and eight Ugandan viruses induced syncytia in primary isolation cultures as well as on first and second passage in PBMC (Table 3). All other viruses were nega-

TABLE 3. CYTOPATHIC CHARACTERISTICS OF HIV-1 WHO NETWORK ISOLATES IN PRIMARY CULTURE AND DURING EARLY PASSAGE IN PBMC

Country	Genetic subtype HMA[a]	Number of isolates	Unchanged Syncytia +	Unchanged Syncytia −	Changed − → +	Changed + → −
Brazil	B	12	2	3	5	2
	C	1		1		
Rwanda	A	4		3	1	
Thailand	B	2		2		
	E	11		5	3	3
Uganda	A	3	2	1		
	D	8	6	2[b]		

[a]HMA, heteroduplex mobility assay (see footnote *b* to Table 2).

[b]One isolate showed single cell killing.

TABLE 4. SYNCYTIUM INDUCTION WITH EARLY PASSAGE HIV-1 ISOLATES FROM THAILAND AND BRAZIL

Virus isolate	Replication in PBMC	Cytopathic effect in PBMC[a]			Phenotype based on syncytium induction
		Primary culture	First passage	Second passage	
Thailand					
001 022 023	+	–	S	S	Changing, neg → pos
003 006 011	+	S	–	–	Changing, pos → neg
009 014 024	+	–	–	–	Negative
019	+	–	D	–	Negative
020 026	+	–	s	–	Negative
021	+	–	D + S	–	Negative
Brazil					
018 019 020 021 028	+	–	S	S	Changing, neg → pos
004 026	+	S	–	–	Changing, pos → neg
014 030	+	S	S	S	Positive
003 017	+	–	–	–	Negative
023 025	+	–	s	–	Negative

[a]S, syncytia; D, cell killing; D + S, mixed cytopathic effect; s, small syncytia; –, no cytopathic effect.

tive for syncytium induction throughout the observation period, or showed a changing pattern. Subtype E viruses from Thailand and subtype B viruses from Brazil were particularly prone to change their cytopathic characteristics during early passage in PBMC. These changes are shown in detail in Table 4. Interestingly, changes occurred in both directions, for non-syncytium-inducing in the primary culture to syncytium-inducing on subsequent passage (001, 022, and 023 from Thailand and 018 through 021 and 028 from Brazil) or, conversely, from initially syncytia-inducing to no cytopathic effect (003, 006, and 011 from Thailand and 004 and 026 from Brazil). The results indicate that syncytia induction in PBMC is not unique to subtype B viruses but may be the property of subtype A, D, and E viruses as well. Thus both the syncytia-inducing and non-syncytia-inducing phenotypes occur over several genetic subtypes. Syncytia induction can be used as a phenotypic marker to follow changes in virus populations during early passage of HIV-1 isolates in PBMC.

Correlation of replicative and syncytium-inducing capacity of HIV-1 isolates

Comparison of syncytia inducing capacities in PBMC and MT-2 cells showed that all viruses rated as s/l 3 or r/h induced syncytia in both cell systems, irrespective of genetic subtype or geographic origin (Table 5). Among viruses rated s/l 2 or s/l 1, 8 out of 27 exhibited syncytia induction in PBMC but not in MT-2 cells. That some of the slow/low viruses can induce syncytia has been observed previously with genetic subtype B viruses.[3] We now confirm and extend this finding to include viruses of subtype A, D, and E.

TABLE 5. CORRELATION OF REPLICATIVE AND SYNCYTIUM-INDUCING CAPACITIES OF HIV-1 ISOLATES OF DIFFERENT SUBTYPES

Genetic subtype	Phenotype[a]					
	s/l 1 NSI		s/l 2 NSI		s/l 3 or intermediate SI	r/h SI
	Syncytium induction in PBMC[b]					
	–	+	–	+	+	+
A	1		3	2	1	
B	2		4	2	5	
C			1			
D			1	1	1	5
E		1	7	2		

[a]SI/NSI refers to replication and syncytium induction in MT-2 cells.
[b]Observed at second passage level.

Correlation of biological phenotype to V3 sequence

The charge of the entire V3 loop (Fig. 2A) or a 15 amino acid region bordering the tip of the loop (Fig. 2B) was compared to the slow/low and rapid/high replicative capacity of HIV-1 isolates. There was a strong association between increased positive charge and the rh phenotype, particularly when examining the 15 amino acid tip of the loop (Fig. 2B).

DISCUSSION

The aim of the present study was to compare markers for the biological phenotype of HIV-1 across genetic subtypes present in four distinct geographic areas. The virus isolates obtained from Brazil, Rwanda, and Thailand showed the slow/low replication pattern, Rwanda and Thailand s/l 1 and s/l 2, Brazil s/l 2 and s/l 3. If the occurrence of slow/low viruses early in HIV infection is a general rule, this was to be expected, since most samples from these three countries were collected within 2 years from seroconversion. For Ugandan subjects, seroconversion time was in most cases unknown. In the four cases where seroconversion time could be calculated, only one sample could be considered as obtained early in HIV-1 infection. As has been demonstrated for HIV-1 of subtype B[8,10,11] the replicative capacity of viruses obtained from subjects with progressive HIV-1 disease is influenced by the duration of infection. In this present study, most viruses isolated around 2 years from seroconversion or later were recovered within a few days of initiation of culture and replicated more readily than viruses isolated earlier during HIV-1 infection (Fig. 1). This suggests a pathogenesis-related increase in replicative capacity of viruses with diverse genetic subtypes over time. Recently, the biological characterization of 23 HIV-1 isolates of genetic subtype E from Thailand has been described.[18] Seroconversion time was unknown and six individuals had a symptomatic HIV-1 infection. The viruses readily replicated in PBMC and in five cases (three from AIDS patients) also in cell lines. This further indicates a strong link between the time of infection and viral phenotype in individuals with increasing severity of HIV-1 infection, now including subtype E as well.

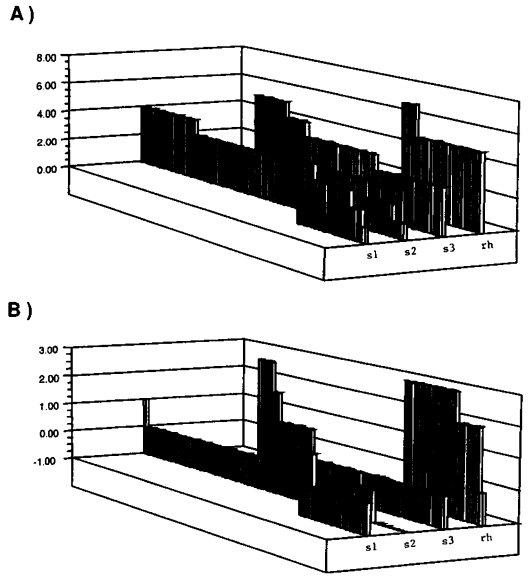

FIG. 2. Charge of the V3 loop and its relationship to the growth characteristics of viral isolates in cultured cells. The net charge on the V3 loop was calculated and is shown for the whole V3 loop. C-to-C inclusive (A) or the region including 13 amino acids bordering the tip of the loop (B) four amino acids on the N-terminal side of GPGR, GPGR, or its analogue, and seven amino acids on the C-terminal side). Each bar represents an individual sequence; in some cases multiple sequences from a single individual are represented. Only sequences generated from viral culture, and not from PBMCs or plasma, were included, to represent the viruses that served as the basis for the phenotype determination as closely as possible. The height represents the net charge on the loop and the rows indicate whether the sequence was from an individual whose viral isolate displayed slow/low (s1, s2, s3) or rapid/high (rh) growth characteristics.

Differences in replicative capacities in primary cultures among slow/low viruses were also observed. HIV-1 isolates from Thailand appeared to replicate more slowly than viruses obtained from Brazil or Rwanda (Fig. 1). Even if viral antigen could be detected within a few days, appreciable amount of virus replication did not occur until several weeks later. In contrast, viruses from Brazil appeared early and replicated without delay (Fig. 1). The time necessary for detection of the first viral antigen-positive culture may depend on the virus load in the HIV-1 infected subject. Estimation of viral load by a semiquantitative PCR suggests that blood samples from Thailand and Brazil do indeed differ in viral copy number (Rübsamen-Waigmann, unpublished). While HIV-1 could regularly be detected in 0.5 μg DNA from Brazilian samples, no HIV-1 DNA could be amplified, even from 1 μg DNA, from Thai samples.

Apart from differences in the patients' viral load, true differences in virus replicative capacities could also be detected. The following parameters were used to characterize virus isolates: (1) the time necessary to reach antigen value OD = 1 in the primary isolation culture, (2) the ability to replicate in cell lines, (3) the ability to induce syncytia in PBMC and/or MT-2 cells. Virus replication in primary isolation cultures, PBMC passages, and cell lines was followed by antigen ELISA and reverse transcriptase assays in parallel. Low antigen values were concomitant with low reverse transcriptase activity across genetic subtypes, hence low antigen values reflect low amounts of virus. By all these criteria Thai viruses generally replicated slowly in PBMC and not at all in cell lines, and rarely induced syncytia. Conversely, Brazilian viruses replicated promptly in primary isolation cultures and in five (out of 13) cases replicated and induced syncytia in MT-2 cells as well (s/l 3/SI). Ugandan viruses were fastest replicating; in most cases the first antigen-positive value was well over OD 1, and syncytia were induced in primary isolation cultures and on subsequent passages in PBMC and in MT-2 cells. To be able to understand the biological significance of the different behavior of Ugandan viruses, work is in progress to collect virus isolates from individuals with known seroconversion times.

Comparison of the different classification systems for biological phenotyping showed that indeed there is an overlap and most of the viruses classified slow/low are NSI, while viruses

classified rapid/high are SI. There is, however, an intermediary group, denoted s/l 3, which is able to replicate and induce syncytia in MT-2 cells but not in any of the other cell lines used in the present study. While replication and syncytia induction in MT-2 cells define the SI phenotype, no replication or only transient replication in cell lines other than MT-2 characterizes the viruses as s/l 3 or intermediate phenotype. Furthermore, syncytia induction in PBMC may be the property of some of the viruses classified as s/l 1 or s/l 2 (Table 5).[3] From the point of view of understanding pathogenesis it is important to recognize that the replicative capacity of HIV-1 isolates is a continuum, where s/l 1, 2, and 3 and r/h denote viruses with increasing replicative capacities. Using the MT-2 test alone allows a rapid estimation of the phenotype of a large number of HIV-1 isolates.

It has been suggested that the phenotype of subtype B HIV-1 isolates from Europe and North America,[19,20] whether they appear as syncytium-inducing (SI) or non-syncytium-inducing (NSI) on MT-2 cells, can be predicted based on V3 sequence.[21–23] Accordingly, positively charged amino acid substitutions at positions 11 and 25 within the loop of variable region 3 (V3 loop) of HIV-1 subtype B envelope have been shown to be associated with the SI phenotype. In an accompanying paper[24] this finding has been extended to HIV-1 of subtype A, D, and E. We now demonstrate that comparison of the biological phenotype of HIV-1 isolates according to the rapid/high and slow/low classification system to the charge of the V3 loop shows a similar strong correlation of the rapid/high phenotype with highly charged V3 loops (Fig. 2).

The use of standard procedures for HIV-1 isolation allowed us to study changes in biological properties of viruses during early passage *in vitro*. The phenotypic marker used was syncytium induction, easily observed in primary isolation cultures as well as during subsequent passages in PBMC and in MT-2 cells. In fact, the majority of virus isolates from Brazil (7 out of 12) and from Thailand (6 out of 11) changed phenotype following isolation. At the same time, Ugandan viruses were stable in their cytopathic characteristics in PBMC. The changes in virus populations imposed by initial passage in PBMC are important to bear in mind when molecular and antigenic studies are performed and results are used to explain pathogenic processes in the patient. Which of these viruses—the virus that corresponds to the major sequence in the patient and may predominate in the early culture, or the virus that readily replicates in PBMC *in vitro*—is important for HIV-1 pathogenesis remains to be clarified.

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