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NOTES

Macrophage-Tropic and T-Cell Line-Adapted Chimeric Strains of Human Immunodeficiency Virus Type 1 Differ in Their Susceptibilities to Neutralization by Soluble CD4 at Different Temperatures

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Molecular clones of three macrophage-tropic and three T-cell line-adapted strains of human immunodeficiency virus type 1 (HIV-1) were used to explore the mechanism of HIV-1 resistance to neutralization by soluble CD4 (sCD4). The three macrophage-tropic viruses, each possessing the V3 and flanking regions of JR-FL, were all resistant to sCD4 neutralization under the standard conditions of a short preincubation of the virus and sCD4 at 37°C prior to inoculation of peripheral blood mononuclear cells. In contrast, the three T-cell line-adapted viruses, NL4-3 and two chimeras possessing the V3 and flanking regions of NL4-3 in the envelope background of JR-FL, were all sCD4 sensitive under these conditions. Sensitivity to sCD4 neutralization at 37°C corresponded with rapid, sCD4-induced gp120 shedding from the viruses. However, when the incubation temperature of the sCD4 and virus was reduced to 4°C, the three macrophage-tropic viruses shed gp120 and became more sensitive to sCD4 neutralization. In contrast, the rates of sCD4-induced gp120 shedding and virus neutralization were reduced for the three T-cell line-adapted viruses at 4°C. Thus, HIV resistance to sCD4 is a conditional phenomenon; macrophage-tropic and T-cell line-adapted strains can be distinguished by the temperature dependencies of their neutralization by sCD4. The average density of gp120 molecules on the macrophage-tropic viruses exceeded by about fourfold that on the T-cell line-adapted viruses, suggesting that HIV growth in T-cell lines may select for a destabilized envelope glycoprotein complex. Further studies of early events in HIV-1 infection should focus on primary virus strains.

The infection cycle of human immunodeficiency virus type 1 (HIV-1) can, in principle, be broken by interference with the attachment of the virus to its cellular receptor, CD4 (2, 17, 23). One method designed to do this was to introduce competition for the binding of HIV to CD4 with soluble forms of the CD4 molecule (sCD4) (8, 23). However, although initially promising results were obtained in vitro, sCD4 was ineffective in vivo. This was due at least in part to the inherent resistance to neutralization by sCD4 shown by primary HIV-1 isolates grown in peripheral blood mononuclear cells (PBMC) compared with the neutralization sensitivity shown by those T-cell line-passaged viruses on which sCD4 was initially tested (7, 23). The resistance of primary viruses to neutralization by sCD4 is not due to an intrinsic inability of their gp120 molecules to bind sCD4 (3, 4, 21, 30). However, intact virions of primary viruses show reduced binding of sCD4 compared with that of virions of T-cell line-adapted viruses (21, 26), probably because of a decrease in the rate of sCD4 binding (18). Curiously, at least some primary viruses become sensitive to sCD4 after prolonged incubation with this agent at 4°C but not at 37°C (18, 21).

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Using molecular chimeras of a macrophage-tropic primary virus (JR-FL) and a T-cell line-adapted virus (NL4-3), we have previously shown that sCD4 resistance and macrophage tropism can both segregate with the identity of the V3 and flanking regions of the gp120 envelope glycoprotein; introducing the JR-FL V3 region into the NL4-3 envelope (24, 25) or the IIIB envelope (12) creates a macrophage-tropic, sCD4-resistant virus. We have extended those studies to explore whether the macrophage-tropic chimeric viruses become sensitive to sCD4 at 4°C. They do, confirming our previous observations with uncloned primary viruses. Furthermore, the macrophage-tropic chimeras have a density of virion-bound gp120 molecules that is approximately fourfold higher than that of T-cell lines. This is consistent with our view that viruses adapted to growth in T-cell lines have destabilized envelope glycoprotein structures, rendering them abnormally sensitive to neutralization (18, 19).

Virion gp120/p24 ratios are higher for macrophage-tropic HIV-1 strains than for T-cell line-adapted ones. Recombinant proviral clones were constructed by substitutions of fragments from a molecular clone of HIV-1 JR-FL into the full-length, infectious clone of HIV-1 NL4-3, pNL4-3, as previously described (1, 15, 24, 25). Based on the pNL4-3 nucleotide and amino acid numbering, substitutions of HIV-1 JR-FL sequences are as follows: SX (formerly pNFN-SX), nucleotide

TABLE 1. gp120/p24 ratios in macrophage-tropic and T-cell line-adapted chimeras

Expt and virus[a]	Amt (ng/ml) of gp120 in:		Amt (ng/ml) of p24 in:		Virion gp120/p24 ratio	Mean ratio ± SD[b]
	Virion fraction	Soluble fraction	Virion fraction	Soluble fraction		
1						
NL4-3	1.6	2.1	31	13	0.051	0.041 ± 0.012
PX	1.1	5.5	23	4.5	0.048	
MX	0.8	2.6	31	11	0.025	
SM	1.9	5.9	7.9	<5.0	0.25	0.17 ± 0.06
SX	5.0	4.2	50	28	0.10	
SP	0.9	7.5	5.1	<5.0	0.17	
2						
NL4-3	1.8	2.4	27	ND[c]	0.067	0.053 ± 0.024
PX	1.4	4.0	18	ND	0.076	
MX	0.61	2.2	38	ND	0.016	
SM	2.3	4.0	8.5	ND	0.27	0.23 ± 0.05
SX	4.3	2.5	29	ND	0.15	
SP	2.4	5.4	9.2	ND	0.26	

[a] NL4-3, PX, and MX are T-cell line-adapted chimeras; SM, SX, and SP are macrophage-tropic chimeras.
[b] Mean ratio values are by category, i.e., for T-cell line-adapted chimeras and for macrophage-tropic chimeras.
[c] ND, not done.

(nt) 6822 to 8887 and amino acid (aa) 202 to 854; SM (formerly pNFN-SM), nt 6822 to 7305 and aa 202 to 362; SP (formerly pNFN-SP), nt 6822 to 7652 and aa 202 to 482; MX (formerly pNFN-MX), nt 7306 to 8887 and aa 363 to 854; and PX (formerly pNFN-PX), nt 7653 to 8887 and aa 483 to 854. The SX, SM, and SP strains contain the V3 and various flanking sequences derived from HIV-1 JR-FL and enter macrophages efficiently compared with the MX, PX, and NL4-3 strains (24, 25). Virus stocks were generated following electroporation of phytohemagglutinin (PHA)-stimulated PBMC with full-length, proviral clones, as described previously (5, 24).

Using HIV-1 stocks prepared from transfection culture supernatants, we found that we were unable to obtain reliable data for sCD4-induced gp120 dissociation, even with well-characterized viruses such as NL4-3. This may be related to the large proportion of defective viruses reported to be present after transfection (14). Typically, we found the infectivity/p24 ratio in the transfection-derived stocks to be from 1 to 10% of that observed with virus stocks derived from culture in PBMC. We therefore focused on the latter stocks.

To obtain a measurement of the proportion of total gp120 that was virion associated, culture supernatants containing the T-cell line-adapted and macrophage-tropic viruses were fractionated on a precooled gel filtration column containing Sephacryl S-1000 to separate virions from free gp120, essentially as described previously (18, 21, 22). The column eluates were inactivated with Tris-buffered saline containing 1% Empigen nonionic detergent (to minimize interference with the p24 assay), 1% fetal calf serum, and, in some experiments, 1 μg of sCD4 (Biogen) per ml. The latter reagent was added to ensure that all samples contained saturating concentrations of sCD4 prior to the assay of gp120 content by enzyme-linked immunosorbent assay (ELISA) whenever sCD4 was an experimental variable. The ELISAs for gp120, using CHO gp120 (BH10) as a reference standard, and for p24, using recombinant p24 (American Biotechnologies Inc.) as a reference standard, were performed as described previously (18, 21, 22). In both experiments whose results are shown in Table 1, the gp120/p24 ratios of the virion fractions were on average approximately fourfold greater for the macrophage-tropic viruses than for the T-cell line-adapted ones. We interpret this to mean that the average density of gp120 spikes on the particles of the macrophage-tropic viruses is significantly greater than that on those of the T-cell line-adapted viruses. This observation will, however, require confirmation by nonbiochemical methods, such as electron microscopy.

Macrophage-tropic HIV-1 chimeric viruses shed gp120 more slowly than do T-cell line-adapted viruses at 37°C. Using stocks of the six chimeric viruses grown in PBMC, we measured the rates of gp120 dissociation from the virions at both 4 and 37°C with or without the addition of 5 μg of sCD4 per ml (Fig. 1). In the absence of sCD4, there was relatively little spontaneous gp120 dissociation from most of the viruses, even during prolonged incubation at either temperature, although we consistently noted significant gp120 loss from PX at 37°C (Fig. 1 and data not shown). This chimeric virus may be inherently more fragile than the others. However, in the presence of sCD4 (5 μg/ml), there was a striking difference in gp120 shedding from the three T-cell line-adapted viruses, NL4-3, MX, and PX, and the three macrophage-tropic viruses, SX, SM, and SP (Fig. 1). At 37°C, there was a marked sCD4-induced gp120 loss from the T-cell line-adapted chimeras, while the macrophage-tropic chimeras showed relatively little propensity to shed gp120 at this temperature. In contrast, at 4°C, all six chimeric viruses shed gp120 slowly over the 8-h duration of the experiment. The net effect is that the T-cell line-adapted and macrophage-tropic chimeric viruses can be clearly distinguished by their responses to sCD4 at different temperatures: the T-cell line-adapted viruses shed gp120 more rapidly at 37°C than at 4°C (Fig. 1a to c); the macrophage-tropic viruses shed gp120 more rapidly at 4°C than at 37°C (Fig. 1d to f).

Macrophage-tropic chimeric strains become sensitive to neutralization by sCD4 at 4°C. The enhanced gp120 shedding shown by macrophage-tropic strains at 4°C suggested that the resistance of these viruses to sCD4 neutralization might be overcome at the lower temperature. We therefore assessed the effects of varying the sCD4 concentration and the duration of the incubation period on the efficiency of neutralization by sCD4 at 4 and 37°C (Fig. 2). Macrophage-tropic and T-cell

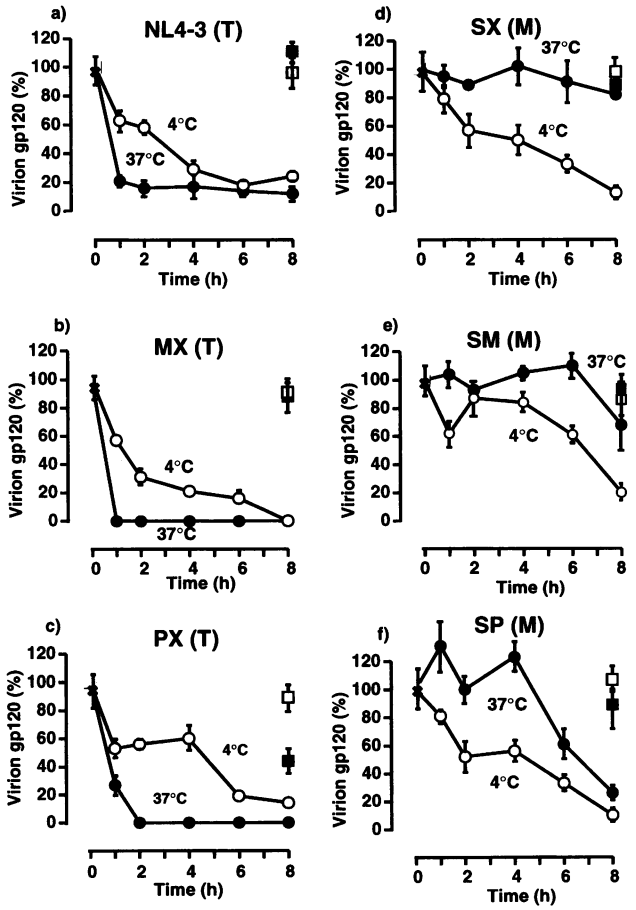

FIG. 1. gp120 loss from T-cell line-adapted and macrophage-tropic chimeric viruses. T-cell line-adapted (NL4-3 [a], MX [b], and PX [c]) or macrophage-tropic (SX [d], SM [e], and SP [f]) viruses were incubated for the times indicated with (○ and ●) or without (□ and ■) 5 μg of sCD4 (Biogen) per ml (9) at either 4°C (○ and □) or 37°C (● and ■). At each time point, the amount of virion-bound (V) and soluble (S) gp120 was determined following fractionation on a precooled gel filtration column containing Sephacryl S-1000 and inactivation of column eluates. The gp120 ratios (calculated as [v/v+s] × 100) at the zero time point (X) for each of the viruses were defined as 100%. The actual gp120 ratios for the viruses were as follows: NL4-3, 21.4%; PX, 13.7%; MX, 11.2%; SX, 50.0%; SP, 17.2%; and SM, 20.9%. V/V+S values obtained at later time points were normalized relative to this 100% value. The datum points presented were each derived from results for a single gel filtration column, with the ELISA determination of gp120 being performed in triplicate. The error bars represent the means ± standard deviations of the ELISA determinations. The pattern of data was shown to be reproducible in two other experiments of similar design.

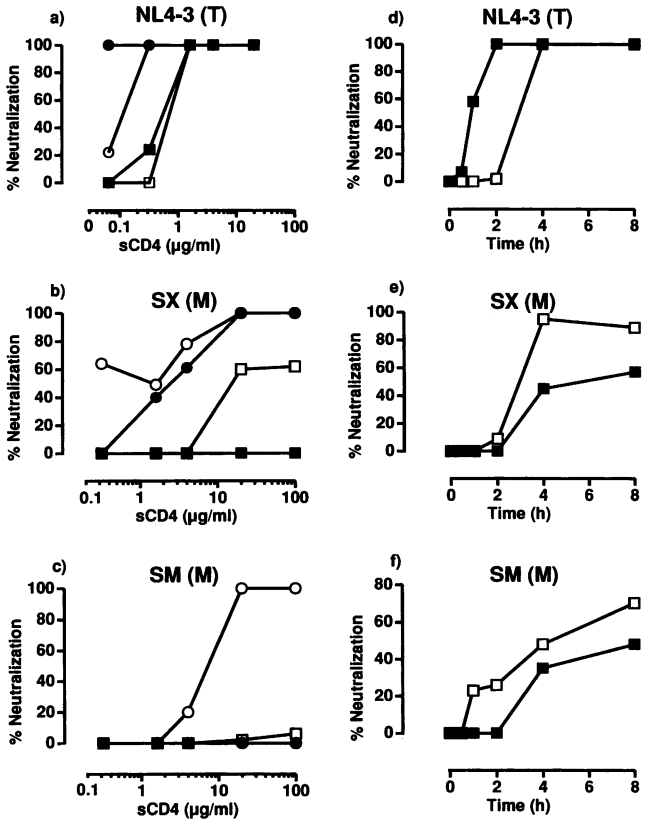

FIG. 2. sCD4 neutralization of T-cell line-adapted and macrophage-tropic chimeric viruses. Neutralization assays were performed essentially as described previously (7, 24), using diluted virus supernatant (100 50% tissue culture infective doses in 100 μl) of NL4-3 (a), SX (b), or SM (c) incubated in duplicate with 0, 0.064, 0.32, 1.6, 4, 20, or 100 μg of sCD4 per ml for 1 h (□ and ■) or 8 h (○ and ●) at 4°C (○ and □) or 37°C (● and ■) prior to inoculation of 10^6 PHA-stimulated PBMC, which were cultured overnight at 37°C. To determine the time course of sCD4 neutralization, the virus was incubated alone or with 0.3 μg of sCD4 per ml for NL4-3 (d) or 3 μg of sCD4 per ml for SX (e) and SM (f) for 0.5, 1, 2, 4, or 8 h prior to the addition to 10^6 PHA-stimulated PBMC. The cells were then washed and cultured in RPMI 1640 medium containing 20% fetal calf serum and 10,000 U of interleukin-2 (Amgen) per ml without further addition of sCD4. The medium was changed on days 1 and 4, and the p24 antigen levels in the sCD4-treated cultures on day 7 were compared with those in an untreated culture. The data are expressed as percent neutralization relative to the samples in the control wells lacking sCD4. Each datum point represents the mean of duplicate samples. The experiments whose results are shown are representative of three of similar design.

line-adapted viruses were incubated with various concentrations of sCD4 for 1 h at either 4 or 37°C before the addition to PHA-stimulated PBMC. At 37°C, the T-cell line-adapted strain NL4-3 was neutralized at relatively low sCD4 concentrations (Fig. 2a), the 90% inhibitory dose being around 0.1 μg/ml, whereas the macrophage-tropic strains SX and SM required much higher concentrations of sCD4 for neutralization (90% inhibitory dose of >100 μg/ml in a 1-h preincubation) (Fig. 2b and c). This is consistent with our previously reported results (24). Increasing the preincubation period of the virus and sCD4 from 1 to 8 h reduced the sCD4 dose required for neutralization, suggesting that the neutralization process was not complete within 1 h (Fig. 2). This effect was especially pronounced for the macrophage-tropic virus SX. At 4°C, there was a modest decrease in the neutralization sensitivity of NL4-3 compared with that at 37°C, but the macrophage-tropic strains SX and SM became much more sensitive to sCD4 at the lower temperature, especially when the incubation period was extended to 8 h (Fig. 2). These neutralization data are entirely consistent with the slow sCD4-induced shedding of gp120 from the macrophage-tropic strains that occurs at 4°C (Fig. 1).

Similar differences between the macrophage-tropic and T-cell line-adapted viruses were observed when the incubation

period for the virus and sCD4 was varied at a constant sCD4 concentration (Fig. 2d to f). There was consistently more rapid neutralization of the macrophage-tropic viruses at 4°C than at 37°C, whereas the converse was observed for the T-cell line-adapted virus NL4-3. In the absence of sCD4, none of the virus strains tested lost much infectivity over 8 h (data not shown), suggesting that interactions of the viruses with sCD4 were responsible for the differences in neutralization rates at the two temperatures. Thus, macrophage-tropic HIV-1 strains can become nearly as sensitive to sCD4 neutralization as T-cell line-adapted strains if a lower temperature and a prolonged incubation period are used instead of the conditions that are standard in neutralization assays: a 30- to 60-min preincubation of the virus and ligand at 37°C before inoculation onto cells and culture at 37°C.

Our results demonstrate that there is a fundamental difference in the responses to sCD4 of T-cell line-adapted and macrophage-tropic chimeric HIV-1 viruses, a conclusion similar to that reached by others (12, 18, 33). The three T-cell line-adapted viruses and the three macrophage-tropic viruses could be clearly separated into two groups according to their reactions to sCD4 treatment at 37 and 4°C. Thus, the macrophage-tropic viruses shed gp120 and were neutralized preferentially at 4°C, whereas the T-cell line-adapted viruses exhibited the same properties preferentially at 37°C. As all six chimeric viruses had been grown in the same cells, PHA-stimulated PBMC, the discrimination between the two groups must be based on genetic, not epigenetic, factors.

Previous studies have suggested that the tropism properties of these chimeric viruses and their abilities to be neutralized by sCD4 at 37°C segregate with the identity of the V3 region and its flanking amino acids; the presence of this V3 fragment from NL4-3 confers both the ability to replicate in T-cell lines and sCD4 sensitivity, and the presence of the fragment of the corresponding region from JR-FL confers macrophage tropism and sCD4 resistance (24, 25). Others using different chimeric viruses have reached a similar conclusion (11, 12, 27). By inference, therefore, the reduced gp120 shedding at 37°C shown by the macrophage-tropic chimeras is also determined by the identities of their V3 regions. We do not, however, believe that the V3 region is the only segment of gp120 that influences HIV tropism or properties such as sCD4 resistance that appear to be linked to it. Other studies, including our own, indicate that changes elsewhere in gp120 or gp41 can have the same effect as manipulation of the V3 region (10, 18, 28, 32, 33). The HIV envelope is very plastic (34), and changes at multiple sites widely separated in the linear sequence may have the same functional effect in the context of the folded, multimeric gp120-gp41 complex.

We suggest that what we have observed in this study with molecularly cloned viruses, and previously with uncloned primary and T-cell line-adapted viruses (18, 28), reflects functional differences between the envelope glycoproteins of macrophage-tropic, primary HIV-1 viruses and those adapted to growth in T-cell lines (15). It has been known for several years that primary HIV-1 isolates grown only in PBMC, or plasma virus ex vivo, resist neutralization by sCD4, in contrast to viruses adapted to growth in transformed T-cell lines (3, 4, 7, 18, 21, 26, 33). All primary viruses we have tested shed gp120 more rapidly at 4°C than at 37°C. The only natural virus strains that shed gp120 rapidly and are potently neutralized by sCD4 at 37°C are those that have been passaged through T-cell lines. In some way, this alters the properties of the viral envelope, probably by selecting for mutations in the envelope glycoproteins that accumulate over several replication cycles, as implied by studies by Willey et al. of variants of HIV-1 ELI (33). The acquisition of full sCD4 sensitivity by two primary viruses required multiple passages through the c8166 cell line (30), and that by another primary virus required multiple passages through the H9 cell line (3); it is not yet known whether prolonged passage of a primary virus through PBMC can select for neutralization sensitivity, but we suspect not.

Our chimeras PX and SX reflect the properties of the parental NL4-3 clone, which was derived from HIV-1 LAI (1), a virus passaged extensively in a T-cell line (31). As primary viruses tend to be able to replicate in both macrophages and $CD4^+$ primary T cells, we are not sure if our results have any relevance to differences between primary viruses that show preference for growth in macrophages and those that grow preferentially in primary T cells. Neither do our results shed any light on the differences between syncytium-inducing and non-syncytium-inducing primary viruses; it is not yet clear what the ability of a primary virus to replicate in the transformed T-cell line MT-2 means under in vivo conditions, other than demonstrating a phenotype capable of more rapid replication under certain conditions. While there are probably unappreciated subtleties in the properties of various categories of primary HIV-1 strains, we believe that any such differences are minor compared with the changes induced on passage of a virus through a T-cell line. We emphasize that the gross changes in the properties of the HIV-1 envelope that occur during HIV-1 selection on T-cell lines are not necessarily reflective of those properties that predispose certain strains to enter those T-cell lines in the first place. Rather, there may be a spectrum of properties reflecting multiple genotypic changes (33).

We interpret the differences between the macrophage-tropic and T-cell line-adapted viruses in the following way. We suspect that at 4°C, most viruses shed gp120 in response to sCD4 at broadly similar rates; this would be consistent with our previous results (18) and those in Fig. 2, although some primary viruses can clearly shed gp120 at 4°C more rapidly than some T-cell line-adapted viruses (21). The true dichotomy is found at 37°C, at which temperature macrophage-tropic or primary viruses shed gp120 very slowly compared with T-cell line-adapted viruses. We suggest that it is the rapid shedding shown by T-cell line-adapted viruses at 37°C, not the resistance of primary viruses, that is the abnormal result; the alternative view is one we are more conditioned to take because of the extensive body of work done on the properties of T-cell line-adapted viruses, but we believe it is the wrong one. Although we have not been able to address this point directly in the present study, our earlier results indicated, but failed to prove unambiguously, that the resistance of primary viruses to gp120 shedding and the neutralization in response to sCD4 at 37°C reflected a very low on-rate for the sCD4-virus binding reaction (18). More work is needed to prove this crucial point, but Willey et al. have demonstrated that increases in both sCD4 binding and gp120 shedding occur when the tropism of HIV-1 ELI is expanded to allow efficient replication in T-cell lines (33). Furthermore, Kabat et al. have shown that the efficiency with which primary viruses could infect HeLa-CD4 cell clones was dependent on the level of CD4 expression, whereas T-cell line-adapted HIV-1 strains infected the HeLa-CD4 clones with an efficiency independent of CD4 expression (13). An interpretation of these data is that the reaction between cell-surface CD4 and the envelope glycoprotein complex of primary viruses is relatively inefficient and rate limiting for infection. This would be consistent with prior results obtained from studies of the interactions of sCD4 with primary viruses (18, 21, 26, 33).

It is now becoming very clear that primary viruses are

relatively insensitive to neutralizing antibodies, which has important implications for vaccine development (6, 16). Our perspective is that the sCD4 resistance of primary viruses is a separate manifestation of the same phenomenon (17, 19); we believe that HIV-1 has evolved an envelope glycoprotein oligomeric configuration that has a relatively unexposed CD4 binding site, and perhaps a poorly exposed V3 loop also, increasing the probability of its survival in the presence of the high concentrations of antibodies to its CD4 binding site that are present in HIV-1-positive sera (20). If this view is correct, sCD4 resistance of primary viruses is nothing but an unfortunate property coincident with a natural antibody resistance mechanism. But what seems clear is that passage of HIV-1 in T-cell lines causes a fundamental genetic alteration in its envelope glycoproteins, perhaps involving destabilization of the gp120-gp41 linkage, that helps to adapt the virus to growth in these cells and, en passant, renders the virus neutralization sensitive. Whether the neutralization sensitivity of T-cell line-adapted viruses is a direct consequence of a reduced density of glycoprotein spikes on a virion's surface requires resolution. Shedding of gp120 is unlikely to be part of the CD4-mediated, HIV fusion pathway (29). However, destabilization of the envelope glycoproteins, leading to an increased gp120 spike loss, may be a surrogate marker for a more subtle change in envelope configuration that increases the efficiency of HIV replication in $CD4^+$ T-cell lines. For example, a genetic alteration that increased the accessibility of the CD4 binding site on gp120 to cell-surface CD4 might be important for promoting HIV entry into T-cell lines, but such a change could simultaneously increase the accessibility of neutralizing antibody (and sCD4) binding sites on the envelope glycoproteins and/or destabilize the entire envelope glycoprotein complex. Notwithstanding the precise mechanism, we suggest that it is not profitable to extrapolate from observations made with T-cell line-adapted viruses, or certain envelope chimeras of such viruses, and predict what might happen with primary viruses under conditions closer to those found in vivo.

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