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CD4, CXCR-4, and CCR-5 Dependencies for Infections by Primary Patient and Laboratory-Adapted Isolates of Human Immunodeficiency Virus Type 1

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We have used a focal infectivity method to quantitatively analyze the CD4, CXCR-4, and CCR-5 dependencies for infections by diverse primary patient (PR) and laboratory-adapted (LA) isolates of human immunodeficiency virus type 1 (HIV-1). Infectivities of T-cell-tropic viruses were analyzed in a panel of HeLa-CD4 cell clones that have distinct quantities of CD4 and in human astroglioma U87MG-CD4 cells that express a large quantity of CD4 and become highly susceptible to infection after transfection with a CXCR-4 expression vector. The latter analysis indicated that PR as well as LA T-cell-tropic viruses efficiently employ CXCR-4 as a coreceptor in an optimal human cell line that contains abundant CD4. Previous uncertainties regarding coreceptor usage by PR T-cell-tropic HIV-1 isolates may therefore have derived from the assay conditions. As reported previously, unrelated LA and PR T-cell-tropic HIV-1 isolates differ in infectivities for the HeLa-CD4 clonal panel, with LA viruses infecting all clones equally and PR viruses infecting the clones in proportion to cellular CD4 quantities (D. Kabat, S. L. Kozak, K. Wherly, and B. Chesebro, J. Virol. 68:2570–2577, 1994). To analyze the basis for this difference, we used the HeLa-CD4 panel to compare a molecularly cloned T-cell-tropic PR virus (ELI1) with six of its variants that grow to different extents in CD4-positive leukemic cell lines and that differ only at specific positions in their gp120 and gp41 envelope glycoproteins. All mutations in gp120 or gp41 that contributed to laboratory adaptation preferentially enhanced infectivity for cells that had little CD4 and thereby decreased the CD4 dependencies of the infections. There was a close correlation between abilities of T-cell-tropic ELI viruses to grow in an expanded repertoire of leukemic cell lines, the reduced CD4 dependencies of their infections of the HeLa-CD4 panel, and their sensitivities to inactivation by soluble CD4 (sCD4). Since all of the ELI viruses can efficiently use CXCR-4 as a coreceptor, we conclude that an increase in viral affinity for CD4 rather than a switch in coreceptor specificity is principally responsible for laboratory adaption of T-cell-tropic HIV-1. Syncytium-inducing activities of the ELI viruses, especially when analyzed on cells with low amounts of CD4, were also highly correlated with their laboratory-adapted properties. Results with macrophage-tropic HIV-1 were strikingly different in both coreceptor and CD4 dependencies. When assayed in HeLa-CD4 cells transfected with an expression vector for CCR-5, macrophage-tropic HIV-1 isolates that had been molecularly cloned shortly after removal from patients were equally infectious for cells that had low or high CD4 quantities. Moreover, despite their substantial infectivities for cells that had only a trace of CD4, macrophage-tropic isolates were relatively resistant to inactivation by sCD4. We conclude that T-cell-tropic PR viruses bind weakly to CD4 and preferentially infect cells that coexpress CXCR-4 and large amounts of CD4. Their laboratory adaptation involves corresponding increases in affinities for CD4 and in abilities to infect cells that have relatively little CD4. In contrast, macrophage-tropic HIV-1 appears to interact weakly with CD4 although it can infect cells that coexpress CCR-5 and small quantities of CD4. We propose that cooperative binding of macrophage-tropic HIV-1 onto CCR-5 and CD4 may enhance virus adsorption and infectivity for cells that have only a trace of CD4.

A major complication in understanding the role of human immunodeficiency virus type 1 (HIV-1) in AIDS derives from the enormous diversity of viruses in single patients and from striking differences in envelope glycoproteins and cellular tropisms of virus isolates (9, 20, 29, 47). Early in infection, nearly all HIV-1 is non-syncytium inducing (NSI) in peripheral blood mononuclear cultures (PBMCs) and is able to replicate in macrophages as well as in CD4-positive T cells (58, 61, 64, 65). The latter viruses are classified as macrophage tropic. Late in disease as the immune system degenerates, T-cell-tropic viruses that cannot grow well in macrophages cultured in standard conditions and that usually are syncytium inducing (SI) become more prevalent, and some of these can also grow in a few CD4-positive leukemic cell lines (4, 31, 52, 55, 56, 58). These primary patient (PR) T-cell-tropic viruses often mutate after several weeks in cultures to form more rapidly replicating and therefore more widely used laboratory-adapted (LA) variants (44, 57, 62). Recently, it has become evident that LA isolates differ substantially from their progenitor patient viruses. LA isolates grow in an expanded repertoire of CD4-positive leukemic cell lines (41) and are much more sensitive to inactivations by soluble CD4 (sCD4) and by many monoclonal antibodies that react with gp120-gp41 envelope glycoprotein complexes (14, 31, 34, 36, 41, 53).

All known HIV-1 isolates can use CD4 as a receptor, but

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some cells that lack CD4 can be infected (40, 54). In addition, coreceptors are also necessary for HIV-1 infections (3, 12, 15–17, 19). Recent studies have indicated that macrophage-tropic viruses employ CC chemokine receptor 5 (CCR-5) as a principal coreceptor (3, 12, 15–17), although several of these viruses can also less efficiently use CCR-3, which occurs principally in eosinophils and is absent from lymphocytes (12). In contrast, LA T-cell-tropic viruses can employ the CXC chemokine receptor 4/fusin/LESTR (CXCR-4) (19). It is not yet certain whether CCR-5 is the only coreceptor in macrophages for macrophage-tropic HIV-1 (51). Moreover, previous studies have not established the coreceptor specificities of PR T-cell-tropic viruses. It has been reported that one T-cell-tropic PR virus (ELI1) could only weakly employ either CXCR-4 or CCR-3 (12). Conceivably, the previous deficiencies in analysis of T-cell-tropic PR viruses could derive from their low infectivities for most cell lines (4, 41, 44). Alternatively, they could possibly employ an unidentified coreceptor.

Differences in sCD4 sensitivities of LA and PR viruses have been extensively analyzed (14, 35, 37, 43). It appears that LA viruses are much more sensitive to inactivation by sCD4 at 37°C and that sCD4 induces a loosening of gp120-gp41 associations that can result in gp120 shedding from virions (35, 38, 39) and in exposure of previously shielded epitopes in these glycoproteins (48–50). In addition, LA virions often spontaneously shed gp120 even in the absence of sCD4 at a faster rate than PR virions (33, 35, 41, 60). Although gp120s extracted from LA and PR virions do not uniformly differ in sCD4 affinities (36, 37, 57), some evidence has suggested that sCD4 affinities of virion-associated gp120 are much lower for PR viruses than for LA viruses (37). These results have implied that gp120-gp41 bonds are tighter in PR viruses and that these bonds shield gp120 and gp41 epitopes and constrain the CD4 binding site of gp120, resulting in virions that have low affinities for sCD4 and reduced reactivities with inactivating antibodies (5, 37, 60, 63).

Previously, we studied T-cell-tropic HIV-1 infections by using a panel of HeLa-CD4 cell clones that differs by 2 orders of magnitude in cell surface densities of CD4 (26). Interestingly, we found that LA viruses are equally infectious for cells with trace or large amounts of CD4, whereas PR viruses infect the cell clones approximately in proportion to CD4 levels (26). Furthermore, infections by both LA and PR viruses appeared to be limited by an inefficient adsorption process. As shown by serial transfers of virus-containing medium onto replica cultures, the infectious titers were only slightly reduced by 2-h incubations with even large numbers of CD4-positive cells, and most of the potentially infectious virus eventually was eliminated from the medium by spontaneous inactivation rather than by cellular adsorption (26, 45). These results suggested that factors which would increase virus adsorption onto cultured cells would enhance growth of HIV-1 and that LA virions may have been selected for higher CD4 affinities.

A deficiency in our previous analysis of the HeLa-CD4 panel derived from use of unrelated LA and PR viruses that presumably differed throughout their genomes. This prevented mapping of viral sequences critical for infection of the HeLa-CD4 panel and precluded molecular level analysis of the factors that control adsorption and infection of T-cell-tropic LA and PR viruses. To address these issues, we have now used the HeLa-CD4 panel to analyze a molecularly cloned infectious T-cell-tropic PR virus and six of its LA variants that can grow to different extents in CD4-positive cell lines and that differ only at known positions in their envelope glycoprotein sequences (22, 60). Our results confirm the previous report (26), establish that *env* gene mutations are responsible for the differences in infectivity of these viruses on the HeLa-CD4 cell panel, and suggest that laboratory adaptation can occur in a graded manner by a series of mutations that cause incremental increases in viral affinities for cellular CD4. In addition, we have developed sensitive quantitative methods to measure coreceptor activities and have analyzed the coreceptor specificities and CD4 dependencies of diverse HIV-1 isolates.

MATERIALS AND METHODS

Cells and viruses. HeLa cells and HeLa clones expressing different levels of CD4 were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum. The clone abbreviations and their relative fluorescence units for CD4 expression are as follows: HI-R, 14; HI-Q, 25; HI-K, 71; HI-P, 128; and HI-J, 234 (26). African green monkey CV-1 cells and human astroglioma U87MG cells were from the American Type Culture Collection (Rockville, Md.) and were maintained in DMEM and in minimal essential medium with 10% fetal bovine serum, respectively. They were made CD4 positive by transduction with the SFF-CD4 retroviral vector as described previously (26). A clone of CD4-positive CV-1 cells was isolated, whereas the U87MG-CD4 cells used were a population that was approximately 80% CD4 positive, with approximately half of the latter being high expressers as seen by immunofluorescence microscopy. Wild-type ELI1 and its mutant derivatives ELI2, ELI3, ELI4, ELI5, and ELI6 have been described elsewhere (22, 44, 60). The plasmid for the ELI7 mutant was constructed by substituting the region that contains the gp41 leucine zipper mutation (E49G) present in variants ELI3 and ELI4 for the corresponding wild-type region of ELI1. Stocks of these viruses were prepared by transfecting HeLa cells with full-length proviral DNA by the calcium phosphate precipitation method (24). Culture media were collected 72 h after transfection, passed through a filter (0.45-μm pore size), aliquoted, and stored at −80°C. Virus stocks from an early passage of the PR isolate 208K4 (K4) and a stock of the LA isolate NL4-3 were generous gifts of Bruce Chesebro (Rocky Mountain Laboratories, Hamilton, Mont.) and were previously described (26). The latter viruses were amplified by brief passage in fresh phytohemagglutinin-stimulated PBMCs. The K4 and NL4-3 viruses were harvested at the peaks of reverse transcriptase activity at day 7. Macrophage-tropic HIV-1 JR-FL and SF162 were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIH) (contributed by Irvin Chen and Jay Levy, respectively). They were grown in PBMC cultures and were harvested from the medium at the peak times of reverse transcription release.

Coreceptor expression. Plasmids encoding CXCR-4 (pcDNA3-LESTR) and CCR-5 (pcDNA3.1-CKR5) were generously provided by Marcel Loetscher (Theodor Kocher Institute, University of Bern, Bern, Switzerland) and John Moore (Aaron Diamond Research Center, New York, N.Y.), respectively. Coreceptor functions were assayed both in transiently transfected cells and in stably transfected cells after selection with G418 (Sigma, St. Louis, Mo.). Calcium phosphate transfections were used for CV-1-CD4 and HeLa-CD4 cells, whereas lipofections with DOTAP (Boehringer Mannheim, GmbH, Mannheim, Germany) were used for U87MG-CD4 cells. In addition, HeLa-CD4 cells (clone HI-J) stably expressing CCR-5 were produced by transduction with the SFF-CCR5 retroviral vector (28) and were highly susceptible to macrophage-tropic viruses.

Assays for virus infectivity and sensitivity to sCD4. Infectivities of viruses on the HeLa-CD4 panel were analyzed by the focal infectivity method (11). Briefly, HeLa-CD4 clonal cells (5×10^3) were plated in a 1-cm^2 well of a 48-well culture plate the day before infection. The cells were pretreated with DEAE-dextran (8 μg/ml; Sigma) in serum-free medium at 37°C for 20 min. The cells were washed once in serum-free medium and then incubated with virus (0.1 ml) at 37°C for 2 h. The viruses were diluted with DMEM with 0.1% fetal bovine serum. The ELI viruses were diluted to the same extent within any single experiment. The K4 and NL4-3 viruses were diluted to give similar relative titers when used to infect HeLa-CD4 clones that have high levels of CD4 expression. After incubations, the viruses were removed, and the cells were incubated with fresh medium at 37°C for 3 days. The cells were fixed in ethanol, and foci of infection were detected following subsequent incubations with HIV immunoglobulin (human) (obtained through the NIH AIDS Research and Reference Reagent Program [contributed by Alfred Prince]), peroxidase-conjugated goat anti-human immunoglobulin G (IgG) (Organon Teknika Corp., Westchester, Pa.), and a substrate solution of 3-amino-9-ethylcarbazole (Sigma). Recombinant sCD4 was obtained through the NIH AIDS Research and Reference Reagent Program (contributed by Ray Sweet). Virus was preincubated with various concentrations of sCD4 at 37°C for 15 min and was then used to infect the HeLa-CD4 cell clone HI-J. To test coreceptor dependencies for infections by HIV-1 isolates, both CXCR-4-expressing U87MG-CD4 and CCR-5-expressing HI-J cells were seeded at 48 h post-transfection. These seedings were at 2×10^4 or 1×10^4 cells/2-cm^2 well of a 24-well culture plate for U87MG-CD4 and HI-J cells, respectively. Assays were performed as described above except that 0.2 ml of diluted virus was used for infections.

TABLE 1. Characteristics of wild-type ELI1 and its variants

Virus[a]	Mutation(s) in *env*[b]		Derivation[c]	Relative susceptibility[d] to sCD4-induced gp120 shedding	Relative binding[e] of CD4-IgG (%)	Expanded tropism[f]		
	gp120	gp41				U937	CEM	H9
ELI1			Wild type	−	6 ± 2	−	−	−
ELI2		M7V	By adaptation	+	29 ± 8	−	++	++
ELI3		M7V, E49G	By adaptation	+	ND[g]	−	++	++
ELI4	G427R	M7V, E49G	By adaptation	+++++	ND	++	+++	+++
ELI5	G427R		By mutagenesis	+++	34 ± 4	−	++	++
ELI6	G427R	M7V	By mutagenesis and adaptation	+++++	56 ± 13	++	+++	+++
ELI7		E49G	By mutagenesis	ND	ND	−	−	−

[a] Wild-type ELI1 virus and the variant viruses ELI2 to ELI7 were produced by proviral DNA transfection of HeLa cells as described in Materials and Methods.

[b] The mutation in gp120 (G427R) occurs in the C4 domain in a region implicated in the binding of gp120 to CD4. The M7V and E49G mutations in gp41 occur in regions found to be important for membrane fusion and in the leucine zipper motif, respectively (18, 21, 23). The α-helical leucine zipper structure has been shown to be important for the oligomerization of gp120-gp41 (7, 46) and for membrane fusion (18, 59).

[c] ELI1, the wild-type virus, was isolated as an infectious molecular clone from a patient with AIDS (2, 44). This PR isolate grew well in PBMCs but had a limited ability to grow in CD4-positive cell lines. Infection of H9 cells by ELI1 resulted in high levels of virus replication only after a delay of more than 25 days. Virus that emerged from this infected H9 culture had an expanded host range and was able to infect, without a delay, T-cell lines H9 and CEM and the promonocytic cell line U937. DNA isolated from infected cells was PCR amplified, and mutations in the gp120 and gp41 genes were identified by sequencing. These PCR products were molecularly cloned into the wild-type ELI1 backbone to produce variants ELI2, ELI3, and ELI4. These are considered to be LA variants because they arose during the growth in cell culture that resulted in expanded tropism. Variants ELI5, ELI6, and ELI7 were created by mutagenesis in order to isolate the contributions of each mutation. ELI6 was subsequently found to also be the major adapted variant selected in U937 cells (60).

[d] Previous analysis showed that the mutations present in ELI2, ELI5, and ELI6 caused increased sensitivity to sCD4-IgG-induced gp120 shedding compared to wild-type ELI1. This increased sensitivity was apparent when assayed at both 4 and 37°C (60, 60a).

[e] Variants ELI2, ELI5, and ELI6 were compared with the wild type for the ability of their virion-associated gp120 to bind CD4-IgG as previously reported (60). Viruses were incubated with CD4-IgG (500 nM) at 0°C for 1 h, and the percentage of virion gp120 associated with CD4-IgG was determined.

[f] Viruses were tested for their abilities to productively infect and to rapidly achieve high levels of replication in the T-cell lines H9 and CEM as well as the promonocytic cell line U937 (22, 60). Tropism was described as being negative (−) if there was no significant peak of reverse transcriptase activity after extensive time in culture. ELI1 and all of the variants were able to infect and replicate in PBMCs.

[g] ND, not done.

RESULTS

Properties of ELI1 virus and its LA variants. ELI1 virus was previously isolated as an infectious molecular clone from plasma of a patient with AIDS after brief expansion in PBMCs (2, 44); it was later used to isolate the LA variants ELI2, ELI3, and ELI4 that grew more efficiently in CD4-positive cell lines (22). The *env* genes of these LA variants were isolated by PCR and were exchanged for the *env* gene of the parental ELI1 molecular clone (22). Because these viruses had several amino acid substitutions in gp120 and gp41, the mutants ELI5, ELI6, and ELI7 were constructed in order to discriminate between the influences of the individual mutations. These full-length proviral DNAs were then transfected into HeLa cells and the rescued viruses were characterized (60), as summarized in Table 1. In general, the mutant viruses showed a variably expanded tropism for CD4-positive leukemic cell lines and correspondingly enhanced susceptibilities to sCD4-induced shedding of gp120. Parental ELI1 virus and its variants contained similar amounts of properly processed gp120-gp41 glycoproteins (60). It is noteworthy that ELI1 caused syncytium formation in PBMC cultures, consistent with some other T-cell-tropic PR viruses from patients with advanced AIDS (44, 55, 56), and that it also grew in Jurkat and MT-4 cells. However, as shown below, ELI1 virus is much less syncytium inducing than its most highly adapted variants.

Infectivities of PR and LA ELI viruses for the HeLa-CD4 panel. In parallel with investigations of the ELI viruses, we analyzed the prototypic T-cell-tropic PR and LA viruses K4 and NL4-3, respectively, which have been previously described (1, 26). This provided an internal control for each experiment and a reference for quantitatively interpreting the ELI results. Figure 1 shows a quantitative analysis (using the immunoperoxidase staining focal infectivity assay [11]) of K4 and NL4-3 virus infections of the HeLa-CD4 panel. Infectivity of PR K4 virus is strongly dependent on CD4 levels of the cells, whereas LA NL4-3 virus infects the clones independently of their CD4 contents above a low trace threshold. Infectious titers of all viruses used in this study were greatly enhanced (approximately 10-fold) by preincubation of the HeLa-CD4 cells with 8 μg of DEAE-dextran per ml, but this did not alter the relative infectivities of any virus for different clones of the HeLa-CD4 cell panel. Thus, the LA and PR titration patterns were unaffected by DEAE-dextran.

Each ELI virus was produced by transfecting its full-length proviral DNA into HeLa cells and by subsequently recovering virus from the culture medium. Figure 2 shows the infectivities of each ELI virus for different clones of the HeLa-CD4 panel. Interestingly, parental ELI1 virus titrates indistinguishably from the prototypic K4 PR virus, whereas the LA variants ELI4 and ELI6 titrate similarly to the LA virus NL4-3. Although ELI2, ELI3, and ELI7 appear to titrate similarly to the PR ELI1 virus, ELI2 seems to be intermediate. Five independent experiments showed that ELI2 is reproducibly at least five times more infectious than ELI1 for HeLa-CD4 clones that have very low CD4 levels, whereas the two viruses are equally infectious on cells with high CD4 levels. This difference between ELI1 and ELI2 is reflected in the best-fit equations for the lines in Fig. 2, which showed the extrapolated intercepts on the infectivity axis to be 8 and 170 for ELI1 and ELI2, respectively. ELI5 also titrates on the HeLa-CD4 panel in an intermediate manner, with a slight degree of CD4 dependence. Similar data were obtained for ELI viruses that had been produced from PBMCs rather than from HeLa cells. These results imply that the individual mutations that contribute to laboratory adaptation have different quantitative effects on CD4 dependence of viral infectivity and that full laboratory adaptation may involve additive effects of several mutations (see Discussion).

sCD4 sensitivity of these PR and LA viruses. Figure 3 shows the sCD4 sensitivities of the prototypic PR K4 and LA NL4-3

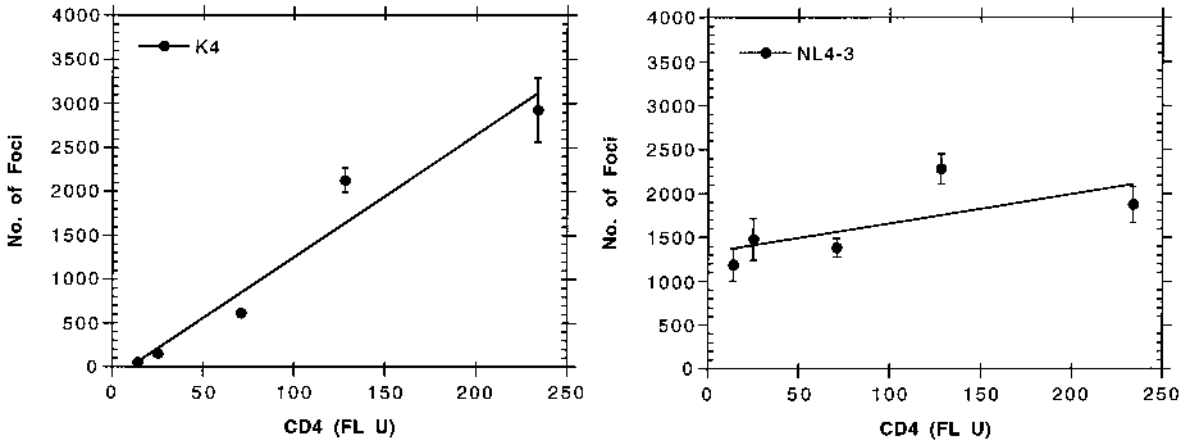

FIG. 1. PR and LA HIV isolates exhibit different infectivity patterns on a HeLa-CD4 panel. The PR isolate K4 and LA isolate NL4-3 were used to infect a panel of HeLa-CD4 clones and were analyzed by the focal infectivity assay as described in Materials and Methods. The HeLa-CD4 clones and their relative CD4 fluorescent (FL) units were as follows: HI-R, 14; HI-Q, 25; HI-K, 71; HI-P, 128; and HI-J, 234. LA NL4-3 virus (right) is equally infectious for cells that have low or high levels of CD4. In contrast, the PR isolate K4 (left) infects the HeLa-CD4 clones in direct proportion to the level of cellular CD4. Each datum point is the mean of triplicate determinations, and the error bars indicate standard errors of the means.

viruses and of ELI1 to ELI7. The viruses were all assayed on the HI-J clone of HeLa-CD4 cells, which contains a large quantity of CD4. The results correlate closely with the infectivity data of Fig. 1 and 2. Thus, ELI4, ELI5, ELI6, and NL4-3 appear to be sCD4 sensitive. ELI2 has an intermediate sensitivity, and ELI1, ELI3, and K4 are resistant to sCD4. Although ELI7 is substantially resistant, it appears to be slightly more sensitive than the PR ELI1 virus.

Syncytium-inducing properties of ELI viruses. HIV-1 isolates are often classified as SI or NSI based on their effects in PBMC cultures (55, 56), and the ELI1 parental virus is clearly SI by this criterion. However, in this study we were able to observe the immunoperoxidase-labeled foci of infection by ELI viruses and to score these foci for numbers of syncytia and for average numbers of nuclei per syncytium as a function of CD4 levels in the HeLa-CD4 clonal panel. When the viruses were titered on HeLa-CD4 cells that had very high CD4 levels, most of the foci of infection contained syncytia. However, as shown by the data in Table 2, when the assays were done on cells with a low CD4 level (i.e., clone HI-R), the fraction of foci that contained syncytia differed dramatically for the different ELI viruses and was strongly correlated with the degree of laboratory adaptation and of sCD4 sensitivity of these viruses. Thus, ELI2, ELI4, ELI5, and ELI6 viruses were much more syncytium inducing than ELI1, ELI3, or ELI7. However, ELI7 virus was clearly anomalous because it was much less syncytium inducing than ELI1, not only in HeLa-CD4 cells but also in PBMCs. Indeed, in PBMCs ELI7 was NSI, although it grew well in the cultures.

CD4 and CCR-5 dependencies of infections by macrophage-tropic HIV-1. Transfections of pcDNA3.1-CKR5 into HeLa-CD4 cells (clone HI-J) resulted in susceptibility to infection by the JR-FL and SF162 macrophage-tropic isolates of HIV-1, as detected by the focal infectivity method (Fig. 4), but reproduc-

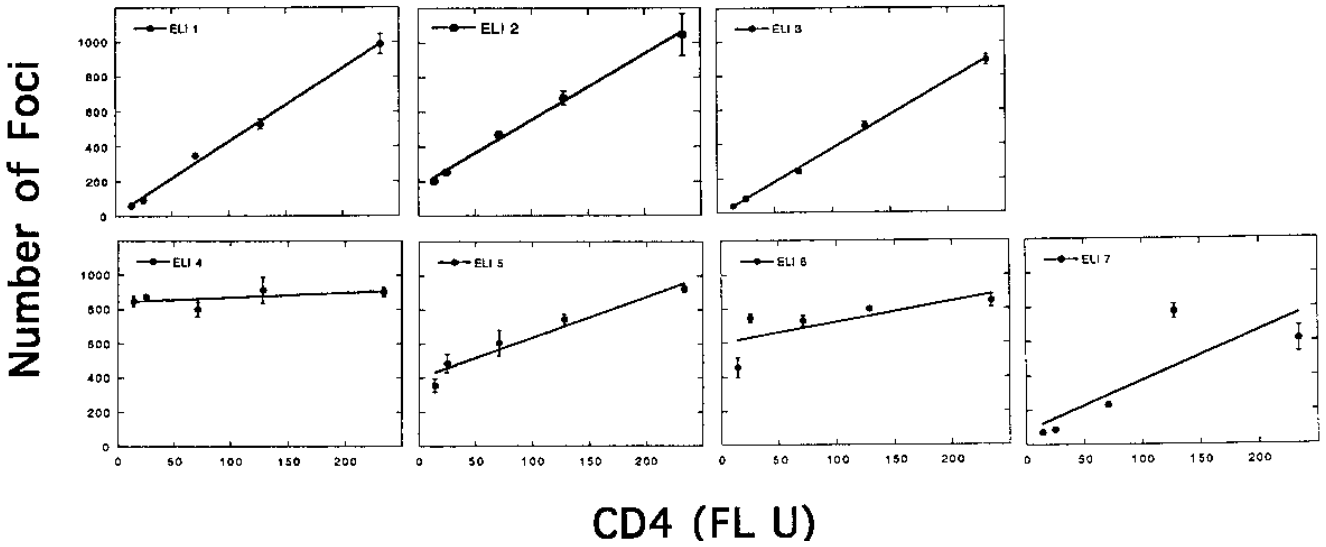

FIG. 2. Infectivities of ELI1 virus and its variants on the HeLa-CD4 panel. The ELI viruses described in Table 1 were used to infect HeLa-CD4 clones and were analyzed by the focal infectivity assay. Each datum point is the mean of triplicate determinations, and the error bars indicate standard errors of the means. FL, fluorescence.

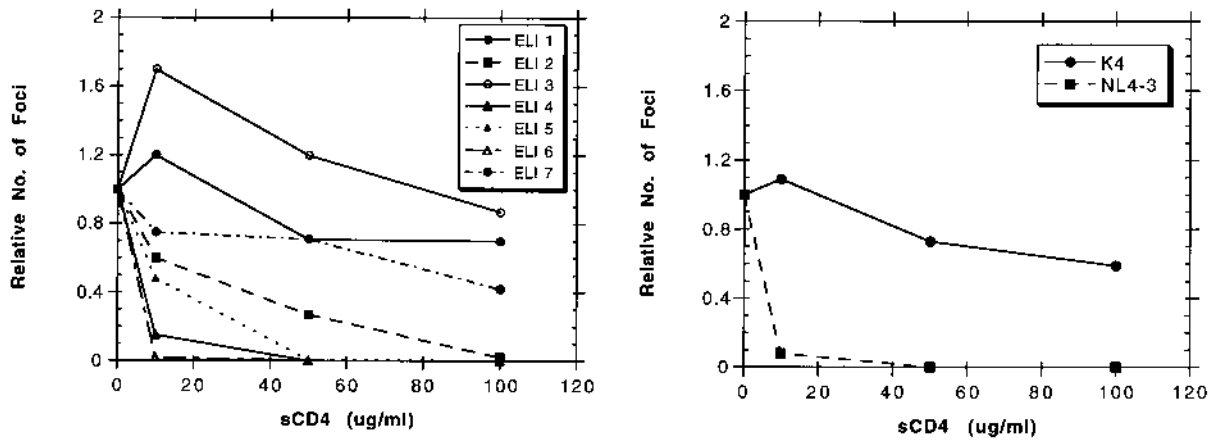

FIG. 3. Sensitivities of PR and LA T-cell-tropic HIV-1 to sCD4. The viruses were preincubated with various concentrations of sCD4 at 37°C for 15 min before infection of the HeLa-CD4 clone HI-J that expresses a large amount of CD4. Infections were quantitatively analyzed by the focal infectivity assay and are expressed as the relative number of foci, with the number of foci without sCD4 being equal to 1.0.

ibly did not alter the titers obtained for the LA and PR T-cell-tropic viruses NL4-3 and ELI1, respectively. Although most foci of macrophage-tropic HIV-1 infections contained only single cells that contained HIV-1 antigens, small syncytia were also observed. No infection by macrophage-tropic HIV-1 occurred in untransfected HeLa-CD4 cells or in HeLa-CD4 cells that were transfected with pcDNA3-LESTR (data not shown).

By performing this same analysis with HeLa-CD4 clones that express distinct amounts of CD4, we were able to determine the CD4 dependencies for infectivity by macrophage-tropic isolates of HIV-1. As shown in Table 3, transfection of the HeLa-CD4 clones with pcDNA3.1-CKR5 was absolutely required for susceptibility to the macrophage-tropic HIV-1 isolates JR-FL and SF162 but had no effect on infectivities of the T-cell-tropic viruses. Although the derivations of the JR-FL and SF162 viruses suggest that they are PR isolates (8, 27), their titers on the three HeLa-CD4 clones used in Table 3 were reproducibly identical within experimental error. In contrast, titers of the PR T-cell-tropic virus ELI1 were highly dependent on cellular CD4 levels in the same experiment. Consistent with Fig. 1, the titers of NL4-3 were independent of CD4 levels.

The results presented above suggest that patient T-cell-tropic viruses are highly dependent on cellular CD4 levels, whereas the macrophage-tropic patient viruses appear less dependent on CD4 and more able to infect cells that have low amounts of CD4. However, it has been reported that macrophage-tropic viruses are substantially resistant to inactivation by sCD4. As shown in Fig. 5, the macrophage-tropic HIV-1 grown in our laboratory were also substantially more resistant to sCD4 than the LA isolate NL4-3. However, as previously reported, SF162 was approximately three to five times more sensitive to sCD4 than JR-FL (25). For T-cell-tropic HIV-1, there is a close correlation between CD4 dependency of infection and resistance to sCD4 (Fig. 1 to 3), but this does not apply to macrophage-tropic viruses in the conditions of our experiments.

CXCR-4 as an efficient coreceptor for PR and LA T-cell-tropic HIV-1. To determine whether CXCR-4 could function as a coreceptor for HIV-1 isolates, we prepared a clone of monkey CV-1-CD4/CXCR-4 cells that had been stably transfected with pcDNA3-LESTR. When these cells were transiently transfected with full-length proviral DNAs for the ELI viruses and for the LA NL4-3 virus, highly significant formation of syncytia occurred above background levels in all of the cultures. This result suggested that CXCR-4 could mediate membrane fusion reactions by all of our T-cell-tropic viruses. Although the levels of syncytium formation were much lower than the levels seen in similarly transfected HeLa-CD4 cultures, this could conceivably have been caused by low transfection efficiencies or proviral expression levels in the monkey cells. We also infected the CV-1-CD4/CXCR-4 cultures with samples of these same viruses and 72 h later identified the foci of infection by immunoperoxidase staining. Although a few infections were unambiguously seen in all of the cultures that had been infected by our T-cell-tropic viruses, the titers were approximately 15 to 100 times lower than the titers measured on HeLa-CD4 cells (clone HI-J) (results not shown). It was

TABLE 2. Syncytium-inducing activities of ELI viruses

Cell type[a] (CD4 FL U)	% of infected foci having syncytia (avg no. of nuclei/syncytium)[b]						
	ELI1	ELI2	ELI3	ELI4	ELI5	ELI6	ELI7
HI-R (14)	16 (4.3)	59 (5.6)	17 (4.3)	82 (8.4)	74 (6.3)	81 (7.2)	2 (3.8)
HI-Q (25)	24 (4.0)	67 (5.7)	26 (4.2)	84 (11.1)	89 (8.0)	83 (10.0)	7 (3.4)
HI-K (71)	50 (5.1)	74 (5.6)	46 (4.8)	78 (7.4)	84 (6.8)	86 (8.2)	7 (3.5)

[a] The HeLa-CD4 clones and their relative CD4 levels as determined by fluorescence (FL)-activated cell sorting analysis were previously described (26). These three clones are the low and medium CD4-expressing clones used for titration of the viruses in the assays shown in Fig. 1 and 2.

[b] Plates of HeLa-CD4 clones that had been infected with the ELI viruses and analyzed by the focal infectivity assay shown in Fig. 2 were examined to determine the syncytium-forming capabilities of the viruses. An infected focus was considered to be a syncytium if it had a multinucleated cell with three or more nuclei. Variants ELI2, ELI4, ELI5, and ELI6 show an increased capacity to form syncytia on these HeLa-CD4 clones. ELI3 had syncytium-forming activity very similar to that of ELI1, whereas ELI7 has a greatly diminished capacity to cause cell-cell fusion. The NSI phenotype of ELI7 was also noted during amplification of virus in PBMCs.

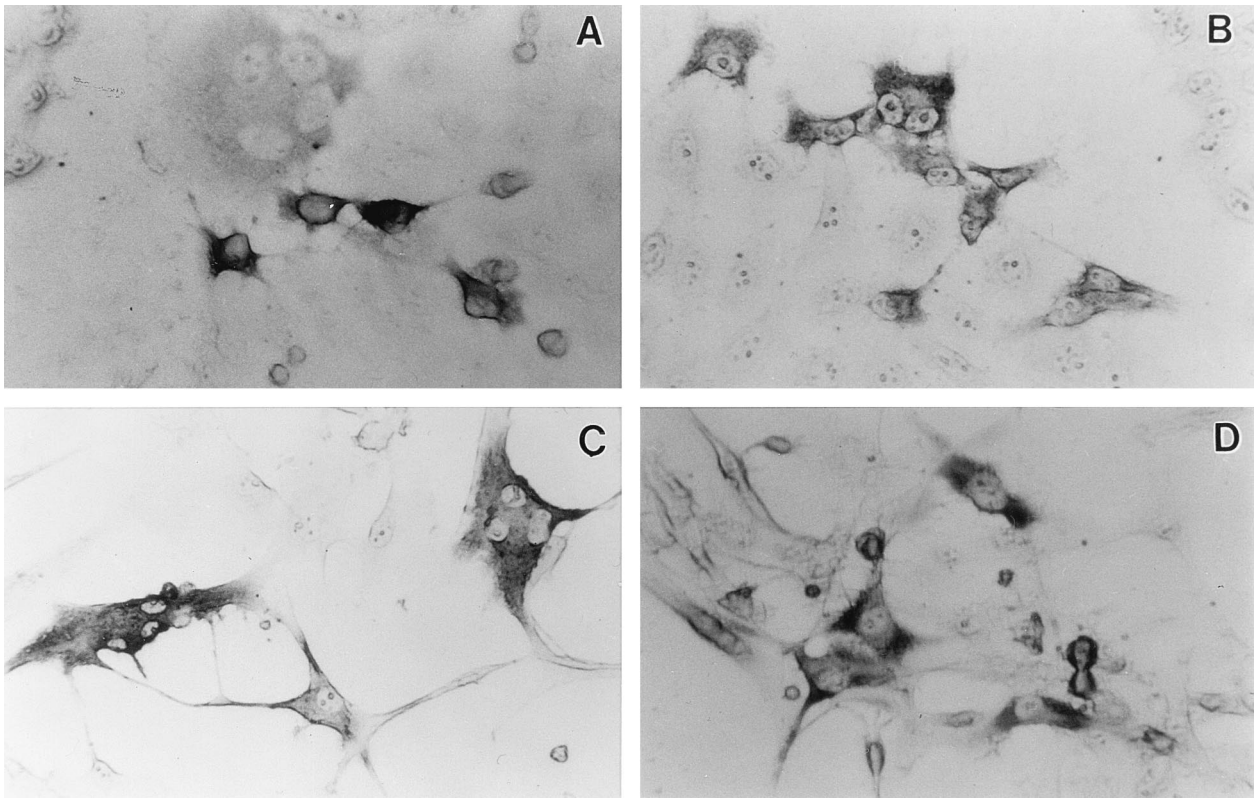

FIG. 4. Immunoperoxide focal infectivity assays of infections by T-cell-tropic and macrophage-tropic HIV-1 in cells that express CXCR-4 and CCR-5, respectively. Shown are CCR-5-expressing HeLa-CD4 cells (clone HI-J) infected with macrophage-tropic JR-FL virus (A) and untransfected HeLa-CD4 cells infected with the PR T-cell-tropic ELI1 virus (B). Panels C and D show U87MG-CD4 cells expressing CXCR-4 that were infected with the LA T-cell-tropic NL4-3 virus and with the PR T-cell-tropic K4 virus, respectively. HeLa-CD4 cells lacking CCR-5 were completely resistant to macrophage-tropic HIV-1, whereas U87MG-CD4 cells lacking CXCR-4 had only a few foci in a large well (Table 4) (results not shown). Cultures are shown at a magnification of ×475.

previously reported that mink cells that coexpress CD4 and CXCR-4 were also inefficiently infected (19).

To further address this issue, we prepared human astroglioma U87MG-CD4 cells that stably express a high level of CD4 (see Materials and Methods) and analyzed their infection after transient transfection with pcDNA3-LESTR. U87MG-CD4 cells were chosen because of their reported resistance to HIV-1 (10). In this cell culture system, all of our T-cell-tropic HIV-1 isolates were highly infectious and CXCR-4 dependent (Table 4 and Fig. 4). Indeed, the titers were only slightly lower than titers of the same virus samples assayed in HeLa-CD4

TABLE 3. Infectivities of macrophage-tropic and T-cell-tropic isolates of HIV-1 in HeLa-CD4 cell clones transiently expressing CCR-5

Cell type[a] (CD4 FL U)	CCR-5[b]	Titer (no. of foci/2-cm^2 well)[c]			
		JR-FL	SF126	ELI1	NL4-3
HI-R (14)	+	116	146	26	1,957
HI-Q (25)	+	130	170	31	1,883
HI-J (234)	+	158	183	441	1,667
	−	0	0	620	1,680

[a] HeLa-CD4 clones and their relative CD4 levels as determined by fluorescence (FL)-activated cell sorting were previously described (21). HeLa-CD4 clones were made susceptible to infection by macrophage-tropic isolates of HIV-1 by transient expression of CCR-5 as described in Materials and Methods.

[b] Cells were transfected with pcDNA3.1-CKR5 or with a negative control plasmid. Infectivities of macrophage-tropic isolate JR-FL and SF162 were completely dependent on CCR-5, whereas infectivities of T-cell-tropic HIV-1 were unaffected by this coreceptor.

[c] Mean of duplicate assays. Macrophage-tropic HIV-1 strains JR-FL and SF162 and T-cell-tropic isolates ELI1 and NL4-3 were propagated as described in Materials and Methods.

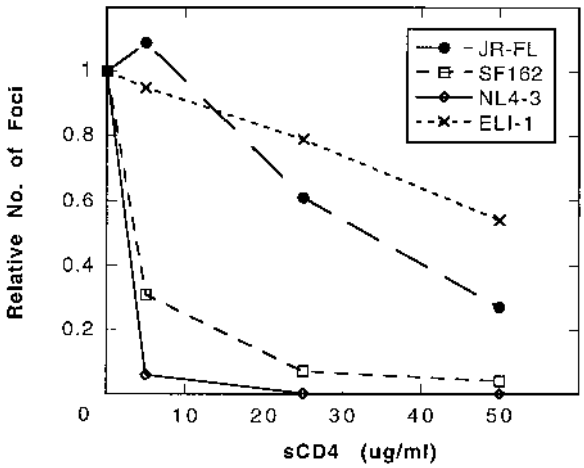

FIG. 5. Sensitivities to sCD4 inactivation of macrophage-tropic and T-cell-tropic HIV-1. The viruses were preincubated with the indicated concentrations of sCD4 for 30 min prior to assay of residual infectivity on HeLa-CD4 cells (clone HI-J). Infectivities for each virus sample relative to the control untreated samples are indicated.

TABLE 4. Infectivities of T-cell-tropic ELI, K4, and NL4-3 viruses on U87MG-CD4 cells that were transiently expressing CXCR-4

CXCR-4[a]	Titer (no. of foci/2-cm^2 well)[b]								
	ELI1	ELI2	ELI3	ELI4	ELI5	ELI6	ELI7	K4	NL4-3
+	72	310	321	329	507	691	134	241	735
−	0	2	1	3	2	2	2	1	0

[a] CXCR-4 was transiently expressed in mixed population of U87MG-CD4 cells as described in Materials and Methods.

[b] Mean of duplicate assays. The titers (number of foci/2-cm^2 well) of these same virus preparations assayed on the HI-J clone of HeLa-CD4 cells were as follows: ELI1, 160; ELI2, 800; ELI3, 1,600; ELI4, 2,000; ELI5, 1,400; ELI6, 1,400; ELI7, 800; K4, 408; and NL4-3, 3,360.

(clone HI-J) cells. This similarity of titers was especially noteworthy because the U87MG-CD4 cell population was heterogeneous and did not uniformly express the transiently transfected pcDNA3-LESTR vector.

DISCUSSION

Efficient use of CXCR-4 as a coreceptor for PR and LA T-cell-tropic HIV-1 and of CCR-5 by macrophage-tropic HIV-1. Our results obtained by using an immunocytochemical assay (11) confirm recent evidence that CXCR-4 can function as a coreceptor for LA isolates of T-cell-tropic HIV-1 and that CCR-5 can serve as the coreceptor for macrophage-tropic isolates. Previous evidence for these conclusions relied on syncytium assays, on expression levels of reporter genes such as the luciferase or chloramphenicol acetyltransferase gene, or on nonquantitative PCR-based assays (3, 12, 15, 16, 19). Because promoters function with distinct efficiencies in different cells, the previous reports could not provide quantitative comparisons of coreceptor activities in mediating infections of different cells. The current studies suggest that CCR-5 can serve as an efficient coreceptor for macrophage-tropic HIV-1 in HeLa-CD4 cells (Table 3) and that CXCR-4 is an efficient coreceptor for T-cell-tropic viruses in U87MG-CD4 cells (Table 4). In contrast, however, monkey CV-1-CD4/CXCR-4 cells are 15 to 100 times less susceptible than HeLa-CD4 cells to infections by T-cell-tropic HIV-1. Moreover, our preliminary results suggest that some cells specifically cannot mediate infections by macrophage-tropic HIV-1 even if they coexpress CD4 and CCR-5 (results not shown). Studies will be needed to learn whether cellular factors in addition to receptors and coreceptors are required for HIV-1 infections.

An additional result of our investigation that was not established by previous evidence is the efficient utilization of CXCR-4 as a coreceptor for infections by PR T-cell-tropic viruses as well as by their LA derivatives (Table 4). Since infections by T-cell-tropic PR viruses are highly dependent on CD4 contents of the cells (Fig. 1 and 2 and Table 3), successful assay of these viruses required use of cells that had a high CD4 content and that were lacking endogenous CXCR-4. These requirements were satisfied by use of human astroglioma U87MG cells that we transduced to a high multiplicity with the retroviral vector SFF-CD4 (26) (see Materials and Methods). After transient transfection with pcDNA3-LESTR, this population of U87MG-CD4 cells became highly susceptible to infections by PR as well as LA T-cell-tropic isolates of HIV-1 (Table 4). Indeed, the titers of these infections were only several times lower than titers of the same viruses on HeLa-CD4 cells (clone HI-J) which contain uniformly high levels of CD4 and CXCR-4. We infer that previous uncertainties concerning coreceptor utilization by PR T-cell-tropic HIV-1 may have resulted from the assays used.

Mechanism of laboratory adaptation by T-cell-tropic HIV-1. These studies of the PR T-cell-tropic virus ELI1 and its variants suggest that their distinct patterns of infectivity for the HeLa-CD4 panel derive from differences in their gp120-gp41 glycoproteins and are caused by the primary mutational events of laboratory adaptation. Although some mutations that occur in the LA variants of ELI1 have only small effects on infectivity for the panel, these mutations also have correspondingly slight effects on viral tropism for CD4-positive leukemic cell lines and on sCD4 sensitivities of the viruses (Table 1 and Fig. 2 and 3). Moreover, combinations of mutations often had greater effects than single mutations, suggesting that laboratory adaptation can involve a graded series of steps that have additive or synergistic consequences.

It is instructive to consider the individual mutations that have been implicated in laboratory adaptation of ELI1. The G427R mutation in gp120 clearly has a strong effect on laboratory adaptation, as evidenced both by infectivities for the HeLa-CD4 panel (Fig. 2) and by its influence on sCD4 sensitivity (Fig. 3). This substitution occurs in the C4 region of gp120, which has been previously implicated as a contact site for CD4 (13, 30, 42). The M7V mutation in the fusion peptide region of gp41 (21, 23) contributes slightly to laboratory adaptation, as seen both by the reproducible increase in infectivity of ELI2 compared with ELI1 for HeLa-CD4 clones with low CD4 levels (e.g., Fig. 2) and by its effects on sCD4 sensitivity (Fig. 3). Also, ELI6 has somewhat stronger LA characteristics than ELI5, supporting the idea that M7V in gp41 can act additively with G427R in gp120. The contributions of the M7V mutation to infectivity on the HeLa-CD4 cell panel and to sCD4 sensitivity suggest that this fusion peptide region can interact at least indirectly with the CD4 binding site of gp120. This is mechanistically reasonable because binding to CD4 is believed to initiate a conformational change in the fusion peptide region that enables it to interact with the cellular target membrane (32, 48–50). Our results suggest a reciprocal influence of the fusion peptide sequence on the CD4 binding site, as expected for an allosteric protein. The E49G mutation of gp41 also contributes positively in some contexts to laboratory adaptation. For example, ELI4 reproducibly appears somewhat less dependent on CD4 for infection of the HeLa-CD4 panel than ELI6 (e.g., Fig. 2). In addition, ELI7 appears to have a slight sensitivity to sCD4. However, it is notable that E49G has a weak influence in our assays and that it also appears to have two countervailing properties. Specifically, in the context of M7V, it seems to reduce the LA phenotype, as seen by indications that ELI3 is more PR-like than ELI2 by all criteria presented here and by the fact that ELI1 is more syncytium inducing than ELI7 (Table 2). The E49G mutation occurs in an amphipathic α-helical leucine zipper region of gp41 that influences oligomerization of gp120-gp41 (6, 7, 46) and is important for membrane fusion (18, 59). We conclude that E49G has a strong inhibitory effect on membrane fusion (Table 2). This inhibition is strong when E49G occurs alone in an otherwise PR ELI1 glycoprotein (i.e., ELI1 is SI whereas ELI7 is NSI), but it is negligible in an LA context (i.e., ELI4 and ELI6 are both strongly SI, and ELI3 and ELI2 are also both clearly SI). A reasonable interpretation is that the E49G leucine zipper mutation inhibits but does not block a conformational switch that is needed for membrane fusion, and that M7V and G427R facilitate the switch and thereby overcome the E49G inhibitory effect.

Previous evidence suggests that adsorption onto cultured cells is a limiting factor in infections by HIV-1 (26, 45). Con-

sequently, the different infectivity patterns of PR and LA viruses on the HeLa-CD4 panel (Fig. 1 and 2) presumably reflect a difference in the CD4 dependence for functional adsorption of these viruses onto cell surfaces and a substantially increased efficiency of attachment for LA viruses. Thus, we infer that LA viruses may adsorb efficiently after contact with cell surfaces that have only trace amounts of CD4, whereas PR viruses may often escape from cells or become inactivated unless the surface concentration of CD4 is very high. This could be due to a slower rate of PR virus binding onto CD4 or to a more rapid rate of its dissociation.

This study and previous evidence (22, 26, 35, 37) strongly suggest that laboratory adaptation of T-cell-tropic HIV-1 principally involves an increased ability of the virus to interact with cell surface CD4 and sCD4, resulting in enhanced infectivity for cultured cells that have a small amount of this receptor (Fig. 1 to 3). Our evidence for efficient use of CXCR-4 but not CCR-5 by all of the T-cell-tropic viruses that we studied (Tables 3 and 4) also strongly suggests that laboratory adaptation of these viruses did not involve a complete switch in their coreceptor specificities. Consistent with this interpretation, the mutations in gp120 and gp41 that contribute to laboratory adaptation do not occur in the V3 loop of gp120 that has been implicated in coreceptor specificity (12). We emphasize that our results cannot exclude the possibility that LA viruses are able to interact promiscuously with coreceptors in addition to CXCR-4 or with other cell surface components that we have not analyzed. However, it is notable that CCR-3, which has also been implicated as an HIV-1 coreceptor (12), is believed to be absent from HeLa cells, lymphocytes, and leukemic T cells (12).

The expanded tropism of LA viruses for CD4-positive leukemic T-cell lines is poorly understood. Abilities of viruses to replicate in such suspension cultures is generally determined by infecting the growing cells and by measuring production of viral proteins or of infectious virions at intervals for several weeks (22, 57, 62). This approach is different from assays such as the focal infectivity assay on adherent monolayers of HeLa-CD4 cells, which measure single cycles of infection (11). If any virus was produced in a suspension culture in low yields or if its efficiency of infection was lower than the cell growth rate, the virus would be scored as nontropic. Since PR viruses infect cultured cells relatively inefficiently, we believe that this could explain their apparent lack of tropism for rapidly growing leukemic T-cell lines despite their abilities to replicate in slowly growing PBMCs.

Syncytium formation correlates with laboratory adaptation and appears to be limited by gp120- and CD4-mediated adhesion of cell surfaces. The PR virus ELI1 is clearly SI based on the criterion that it induces syncytia in PBMC cultures. However, our analysis using the HeLa-CD4 panel provides a more systematic analysis of the syncytium-inducing properties of viruses. As shown in Table 2, when they are assayed in HeLa-CD4 cells that have high CD4 levels, all of the ELI viruses cause some syncytia. However, in cells with low CD4 levels (i.e., the HI-R clone), the ELI viruses differ dramatically in syncytium-inducing capabilities, as seen by both numbers of syncytia and average numbers of nuclei per syncytium. Moreover, their syncytium-inducing activities correlate closely with their LA characteristics, as indicated by sCD4 sensitivities (Fig. 3), infectivities for the HeLa-CD4 panel (Fig. 2), and expanded tropisms for CD4-positive cell lines (Table 1). However, ELI7 is less syncytium inducing than ELI1. This exception can be explained because the mutation in ELI7 alters a fusogenic leucine zipper region of gp41 (18, 59). A simple interpretation of Table 2 is that laboratory adaptation involves an increased affinity of gp120-gp41 complexes for cell surface CD4, and that syncytium formation is limited by gp120-gp41 glycoproteins on one cell binding to CD4 on the surface of an adjacent uninfected cell.

CD4 requirements for infectivity by macrophage-tropic HIV-1. For all known T-cell-tropic PR and LA isolates of HIV-1, there is a very close correspondence between resistance to inactivation by sCD4 and dependency of infectivity on cellular CD4 concentrations (Fig. 1 to 3). This correspondence suggests that these assays measure a common viral property. Other evidence suggests that gp120-gp41 bonds may be tighter in PR viruses and that these bonds constrain the CD4 binding site of gp120 and contribute to sCD4 resistance and low affinity for cellular CD4 (22). In contrast, this correspondence does not apply to the macrophage-tropic viruses JR-FL and SF162, which are partially or fully resistant to sCD4 but highly infectious for HeLa-CD4/CCR5 cells that have only small quantities of CD4 (Table 3 and Fig. 5). Based on these results, we propose that macrophage-tropic viruses may bind strongly to CCR-5 after weakly binding to CD4, and that this could enhance their adsorption and infectivity for cells that have only a trace of CD4. This difference in CD4 dependency for infectivity of HIV-1 isolates is consistent with other evidence that macrophage-tropic isolates efficiently infect cells that express different quantities of CD4 (i.e., CD4-positive T cells and macrophages), whereas PR T-cell-tropic viruses preferentially infect mature T cells that express relatively high levels of CD4.

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The first two authors contributed equally to this work.

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