JOURNAL OF VIROLOGY, Sept. 1997, p. 6509–6516 Vol. 71, No. 9
0022-538X/97/$04.00+0
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Simian Immunodeficiency Virus Variants with Differential T-Cell and Macrophage Tropism Use CCR5 and an Unidentified Cofactor Expressed in CEMx174 Cells for Efficient Entry

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Received 8 January 1997/Accepted 6 June 1997

The recent identification of coreceptors that mediate efficient entry of human immunodeficiency virus type 1 (HIV-1) suggests new therapeutic and preventive strategies. We analyzed simian immunodeficiency virus (SIV) entry cofactors to investigate whether the macaque SIV model can be used as an experimental model to evaluate these strategies. Similar to primary HIV-1 isolates, a well-characterized molecular clone, SIVmac239, which replicates poorly but efficiently enters into rhesus alveolar macrophages and an envelope variant, SIVmac239/316Env, with an ~1,000-fold-higher replicative capacity in macrophages used the β-chemokine receptor CCR5 for efficient entry. The transmembrane portion of 316Env allowed low-level entry into cells expressing CCR1, CCR2B, and CCR3. A single amino acid substitution in the V3 loop of SIVmac239/316Env, 321P→S, impaired the ability to enter into the T-B hybrid cell line CEMx174 but had relatively little effect on entry into primary cells and HOS.CD4 cells expressing CCR5. Although CEMx174 cells do not express CCR5, most SIVmac variants entered this hybrid cell line efficiently but did not enter the parental T-cell line CEM. It seems likely that CEMx174 cells express an as-yet-unidentified, perhaps B-cell-derived cofactor which allows efficient entry of SIVmac.

Infection of rhesus macaques with simian immunodeficiency virus (SIVmac) provides a useful animal model for studying human immunodeficiency virus (HIV) pathogenesis and for testing vaccine strategies (18, 19, 23). The envelope glycoproteins of both HIV and SIV mediate the binding of virus particles to CD4 receptor molecules on the surfaces of target cells (2, 11, 15). It has been known for more than a decade that in addition to CD4, efficient viral entry into target cells requires the participation of other cell surface molecules (2, 37, 43). For HIV type 1 (HIV-1), some of these entry cofactors have recently been identified. T-cell-adapted strains of HIV-1 use the G protein-coupled receptor CXCR4 (also termed 7TMS, fusin, HUMSTR, or LESTR) for efficient entry (4, 22). Primary, macrophage-tropic strains of HIV-1, however, use the CC β-chemokine receptor CCR5 (12, 50, 51), which is expressed in monocytes, macrophages, and primary T cells, as the major entry cofactor (1, 9, 17, 21). CCR5 seems to play an important role in HIV-1 transmission and pathogenesis (16, 28, 36, 45, 51). Some HIV-1 variants can use additional β-chemokine receptors, including CCR2B and CCR3, for entry (9, 20). The HIV-1 envelope glycoprotein V3 loop is critical for the ability to use different coreceptors and for chemokine-mediated blockage of infection (9, 10). The V3 loops of both the HIV and SIV envelopes have previously been shown to be important for determining the viral cell tropism, including the tropism for primary macrophages (3, 6–8, 10, 14, 24, 25, 32, 39–41, 44, 53).

Molecularly cloned SIVmac239 caused AIDS and death within the first 6 months of infection in about 40% of infected rhesus macaques (31). The other 60% of infected animals developed a more protracted course of disease that closely resembles AIDS in humans. Although SIVmac239 is highly pathogenic, it replicated very poorly in primary rhesus macaque alveolar macrophages (39). Some animals developed characteristic encephalitis and giant-cell pneumonia after infection with SIVmac239 (19, 39). In these macaques, macrophage-tropic variants that replicated up to 1,000-fold more efficiently in macrophages evolved (39). A detailed analysis of one of these variants, SIVmac316, revealed that at least five amino acids across the entire length of Env contributed to the enhanced ability to replicate in macrophages (39). None of these changes, however, were located in the V3 loop region (39).

We have analyzed coreceptor usage by a collection of SIVmac variants with highly different replicative capacities in macrophages and some mutants containing specific changes in the V3 loop that had differential effects depending on the cell type. We show that all variants can use CCR5, but not CXCR4, for efficient entry. A single amino acid change in the V3 loop of one SIV variant has little effect on entry into CCR5-expressing cells but impairs entry into CEMx174 cells. Our results indicate that CEMx174 cells express an as-yet-unidentified, perhaps B-cell-derived cofactor which allows efficient entry of SIVmac.

MATERIALS AND METHODS

Plasmids. By PCR, nucleotides 6604 to 7757 of the SIVmac239 *env* gene (numbering of Regier and Desrosiers [47]) were deleted as previously described (48). The second exons of *tat* and *rev*, which overlap with the *env* reading frame, and the Rev-responsive element were not removed. The *nef*/U3 region of the *env* deletion mutant was replaced by a corresponding fragment from SIVΔNU (26)

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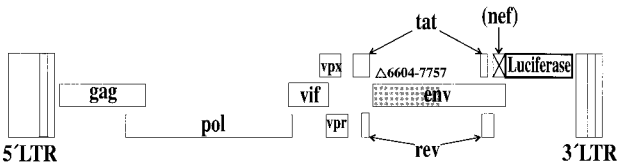

FIG. 1. Modifications in the SIVmac genome to generate the *env*-defective luciferase reporter virus. The deleted area in *env* is shaded; the open reading frames and nucleotide numbers shown are those of SIVmac239 (47). The *nef* gene was replaced by the luciferase gene as described in Materials and Methods.

containing deletions of 513 nucleotides in *nef* and the U3 region. The luciferase reporter gene was amplified by PCR with primers lucs (5′-CCGGGACCATG GAAGACGCCAAAAACATAA-3′) and luca (5′-CCGGTTACAATTTGGA CTTTCCGCCCT-3′) and cloned into the deleted *nef* region, as previously described for the interleukin 2 gene (26), to generate an *env*-deficient SIV luciferase reporter construct, pBR239ΔenvLuc (Fig. 1).

The *Hin*dIII restriction site upstream of the SIVmac *env* gene and the *Eco*RI site just downstream of the viral long terminal repeat (LTR) in the flanking vector sequences were used to clone different *env* genes into the pcDNA3 expression vector (Invitrogen, San Diego, Calif.). As described previously (39), in the molecular clone SIVmac239/316Env, the entire *env* gene is derived from the macrophage-tropic SIVmac316 variant. SIVmac239/316SU contains the outer membrane protein of SIVmac316, SIVmac239/316TM contains the transmembrane protein of SIVmac316, SIVmac 316* contains a truncated transmembrane protein, and SIVmac239MER contains three amino acid changes (67V→M, 176 K→E, and 382G→R) that contribute to macrophage tropism. All the Env expression plasmids used differed only in the specific changes in the viral envelope described previously (32, 39, 40). An overview of the Env variants analyzed is given in Table 1.

To generate an SIV Tat expression construct, the first coding exon of the SIVmac239 *tat* gene was amplified with primers containing *Bam*HI and *Eco*RI restriction sites (p5′TAT, 5′-ATATGGATCCTAGACATGGAGACACCCT-3′, and p3′TAT, 5′-ATCCGAATTCTTATTGTTTGATGCAGAAGATG-3′) and subsequently cloned into the pcDNA3 vector. The absence of mutations in the PCR-derived portion was confirmed by sequence analysis.

***env* complementation assay.** The generation of pseudotyped reporter viruses was essentially performed as previously described (17), except that the *env*-defective SIV luciferase reporter virus described above was used in most assays. Briefly, 293T cells were cotransfected by the calcium phosphate method with 10 μg (each) of the pBR239ΔenvLuc reporter construct and the appropriate Env expression vector. After overnight incubation, the medium was changed, and virus was harvested 24 h later. Viral stocks were frozen in aliquots at −80°C and quantitated by a commercial HIV-1 and HIV-2 enzyme-linked immunosorbent assay (Medipro). Cells were seeded on 24- and 48-well dishes and infected with reporter virus containing 100 ng of p27 antigen in a total volume of 1.0 and 0.5 ml of medium, respectively. After overnight incubation, fresh medium was added, and cell lysates (100 and 50 μl, respectively) were prepared 4 days later. The luciferase activities in 20-μl extracts were measured with commercial reagents (Promega). In some control experiments, the NL4-3-Luc-R$^-$E$^-$ reporter construct (13) and/or HIV-1 Env expression plasmids were used according to the same protocols.

Cell lines. HOS.CD4 cell lines expressing various β-chemokine receptors were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (FCS) and 1 μg of puromycin per ml. The human T-B hybrid cell line CEMx174 and C8166 cells were maintained in RPMI 1640 medium supplemented with 10% FCS and antibiotics. Rhesus and human peripheral blood mononuclear cells (PBMC) were isolated by using lymphocyte separation medium (Organon Teknika Corp., Durham, N.C.) stimulated for 3 days with 2 μg of phytohemagglutinin per ml and cultured in RPMI 1640 medium with 20% FCS and 100 U of interleukin 2 per ml. Mononuclear cells were isolated from fresh blood samples by using lymphocyte separation medium as described above. Cells were seeded on 24-well plates and cultured for 3 days in RPMI 1640 medium supplemented with 10% human serum (Gibco-BRL), 50 U of granulocyte-macrophage colony-stimulating factor (Gibco-BRL) per ml, and antibiotics to allow monocyte differentiation and adherence to plates. Nonadherent cells were removed by extensive washing with phosphate-buffered saline on days 3 and 4, and macrophages were used for infection 1 week after isolation.

Fusion assay. A modified CEMx174 cell line containing the gene encoding secreted alkaline phosphatase (SEAP) under the control of a truncated SIV LTR was used to quantitate cell-cell fusion events. The generation of this cell line has been described elsewhere (38). 293T cells were cotransfected with 5 μg (each) of Env and Tat expression plasmids as described above. After overnight incubation, transfected cells were cocultivated with freshly diluted CEMx174-SEAP cells. On the following day, cocultures were investigated for syncytium formation. To quantitate cell-cell fusions, 20-μl aliquots of cell-free supernatant were used to measure the amount of SEAP released in the medium with a commercial kit (Promega).

Detection of β-chemokine receptor mRNAs. Total cellular RNA was prepared with Triazol reagent (Gibco-BRL) and treated with RNase-free DNase I (Boehringer Mannheim). Reverse transcription was performed in a 20-μl reaction volume containing 1 μg of total RNA, 1 μg of oligo(dT), 200 U of Superscript reverse transcriptase (Gibco-BRL), and 0.5 mM (each) dATP, dCTP, dGTP, and dTTP. After incubation at 37°C for 1 h, a 2-μl aliquot of the cDNA product was amplified in a 20-μl reaction volume containing 0.1 μg of each primer and 1 U of a *Taq*-Pfu polymerase mixture (Boehringer Mannheim) by using a DNA thermal cycler (Perkin-Elmer Cetus Corp.). The cycler program consisted of initial denaturation at 94°C followed by 30 repeated cycles of denaturation at 94°C for 40 s, primer annealing for 40 s at 60°C, and extension for 1 min at 72°C. To control for the presence of genomic DNA, control cDNA reaction mixtures from which reverse transcriptase was omitted were prepared in parallel. These were uniformly negative (data not shown). PCR products were analyzed on a 1% agarose gel. The respective sequences for the 5′ and 3′ primers used are as follows: glyceraldehyde-3-phosphate dehydrogenase, CCATGGAGAAGGC TGGGG and CAAAGTTGTCATGGATGACC; CCR1, GCCCAGAAACAA AGACTTCACGG and GGAATCCTGGGGAACATAGAAC; CCR2A, ACG CATTTCCCCAGTACATC and TGTGATTCAAACGACATCAT; CCR2B, TGAGACAAGCCACAAGCTG and CTGAATGCGTGAGCCCTTTGCTC; CCR3, TTCTATCACAGGGAGAAGTG and ACTGGAAGTTTGAAGGAC TGTT; CCR4, GCAAGCTGCTTCTGGTTGGGCCCAG and GAATGTGG AAAAGTTCATTGAC; CCR5, GGTGGAACAAGATGGATTAT and ATGT GCACAACTCTGACTG; and CXCR4, AGCGAGGTGGACATTCATC and ACGTGATTCACTACAGCTC.

TABLE 1. Replication of the SIVmac envelope variants analyzed[a]

Virus	Replication of SIVmac variants in[b]:		
	CEMx174	PBMC	Macrophages
SIVmac239	++	++	±
SIVmac239/316ENV	++	++	++
SIVmac239/316SU	++	++	+
SIVmac239/316TM	++	++	+
SIVmac239MER	++	++	+
239/316ENV 321P→S	±	++	++
239/316ENV 325M→I	++	++	++
239/316ENV 321P→S, 325M→I	−	++	+
239/316ENV 326S→P, 328L→F	+	±	−
239/316ENV 332S→L, 337D→V	++	−	+
239/316ENV 324I→L	++	++	++
SIVmac239/316ENV*	++	++	+++

[a] The replicative properties of the molecular SIVmac239 clone, the macrophage-tropic variants SIVmac239/316ENV, SIVmac239/316SU, SIVmac239/316TM, SIVmac239MER, and SIVmac239/316ENV*, and the SIVmac316 V3 variants have been described previously (35, 44).

[b] −, no replication detected; ±, low levels of replication detected in some experiments; + to ++, maximal levels of virus production detected (10 to 50% of those of the original SIVmac239 clone in CEMx174 cells and PBMC and of the SIVmac239/316ENV clone in macrophages).

RESULTS

CCR5 is a major coreceptor for SIVmac variants with differential abilities to replicate in macrophages. An *env* complementation assay was used to assess the efficiencies with which Env glycoproteins that are associated with different replicative properties (Table 1) mediate the entry of SIVmac into HOS.CD4 cells expressing different β-chemokine receptors. Pseudotyped SIVmac was produced by cotransfection of 293T cells with Env expression plasmids and an envelope-deleted SIVmac provirus in which the *nef* gene was replaced by the luciferase gene (Fig. 1). Control viruses lacking envelope glycoproteins were produced by transfecting the proviral pBR239ΔenvLuc plasmid alone. HOS.CD4 cells expressing various seven-transmembrane-segment receptors were used as target cells for infection with virus stocks normalized for equal amounts of p27 antigen. The efficiency of viral entry was assessed by luciferase assay at 4 days postinfection.

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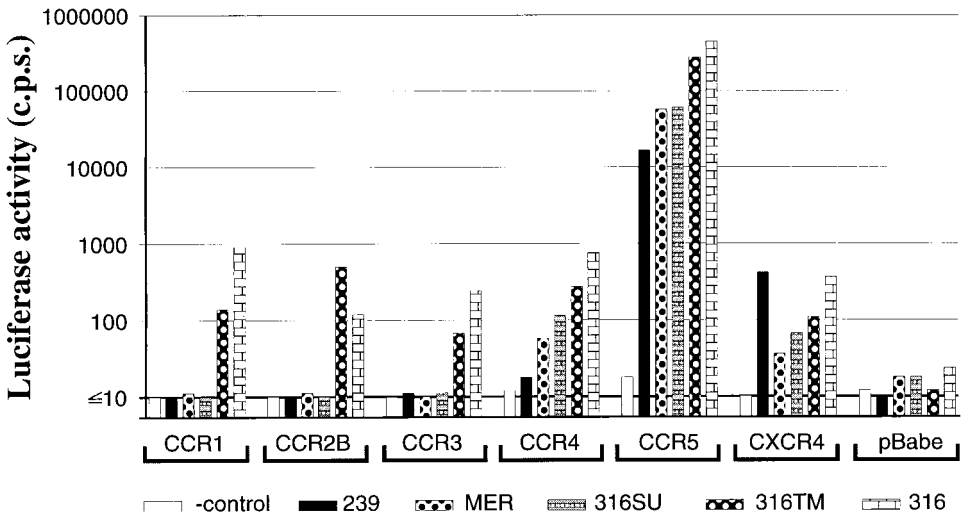

FIG. 2. CCR5 mediates efficient entry of T-cell-tropic and macrophage-tropic SIVmac variants. HOS.CD4 cells stably expressing β-chemokine receptors and the empty pBABE expression vector as indicated were infected with pseudotyped SIV luciferase reporter virus containing 100 ng of p27 antigen. Cells were plated on 24-well dishes and infected the next day in a total volume of 1 ml. After overnight incubation, the cell culture medium was changed, and the luciferase activities were measured 4 days later as described in Materials and Methods. − control, only pBR239ΔenvLuc DNA transfected to generate viral particles. The results for a single experiment are shown. Two independent experiments yielded similar results. After infection with murine leukemia virus-pseudotyped reporter virus, comparable reporter gene activities were observed in all seven HOS.CD4 cell lines.

As shown in Fig. 2, SIVmac pseudotyped with either 239Env, derived from the T-cell-tropic clone, or 316Env, derived from the macrophage-tropic variant, entered CCR5-expressing HOS.CD4 cells with high efficiency. The reporter gene activities obtained after infection of HOS.CD4 cells expressing other CCR receptors or CXCR4 were about 3 to 4 orders of magnitude lower. In contrast, equal levels of luciferase activities were observed after infection of HOS.CD4 cells expressing any of the β-chemokine receptors or CD4 alone with SIV particles pseudotyped with Env derived from murine leukemia virus (data not shown). In the absence of Env, the luciferase values were close to background. In HOS.CD4 cells expressing CCR5, the luciferase values obtained after infection with reporter virus containing 239Env were about 15- to 30-fold lower (24 ± 5.2; obtained in four independent experiments) than those after infection with 316Env-pseudotyped virus. Nonetheless, expression of CCR5, the major coreceptor for macrophage-tropic HIV-1 isolates, conferred about 1,000-times-higher susceptibility for infection with SIVmac239 than did expression of the major coreceptor for T-cell-adapted HIV-1 strains, CXCR4 (Fig. 2). SIVmac reporter virus containing recombinant glycoproteins with gp120 (316SU) or gp41 (316TM) derived from the macrophage-tropic SIVmac316 variant entered CCR5-expressing HOS.CD4 cells with an efficiency between those of SIVmac239 and SIVmac316 (Fig. 2). The gp41 of SIVmac316 consistently conferred slightly higher entry efficiency into CCR5-expressing cells, although the pSIVmac239/316SU virus replicates with higher efficiency in macrophages compared to that of the pSIVmac239/316TM virus (39).

Although CCR5 is clearly a major coreceptor for both SIVmac239 and SIVmac316, less efficient entry was also observed in cells expressing other β-chemokine receptors. Variants containing the transmembrane part of 239 (239Env, 316SU, and MER) showed luciferase values at or close to nonspecific background levels in HOS.CD4 cells expressing CCR1, CCR2B, or CCR3 (Fig. 2). In contrast, SIVmac pseudotyped with Env containing the gp41 derived from SIVmac316 induced about 10- to 100-fold-elevated luciferase activities after infection of HOS.CD4 cells. However, SIVmac239 and SIVmac316 showed comparable low entry efficiencies into CXCR4-expressing HOS.CD4 cells (Fig. 2).

Influence of V3 changes on viral entry. All SIVmac316 V3 loop variants analyzed in this study entered CCR5-expressing HOS.CD4 cells with much higher efficiency than those for cells expressing other β-chemokine receptors (Fig. 3). Some changes in the V3 loop of 316Env that were previously described to have little impact on replication in primary rhesus macaque alveolar macrophages resulted in slightly reduced entry efficiency (321P→S, 321P→S plus 325M→I, 324I→L, and 332S→L plus 336D→V) or had no significant effect (325M→I) (Fig. 3). A double mutant, 326S→P and 328L→F, that was previously found to replicate inefficiently in rhesus alveolar macrophages (Table 1) showed about 100-fold-lower luciferase activities compared to those of 316Env. Some minor but consistent variations were observed in the usage of other coreceptors. For example, the 324I→L variant showed about 5- to 10-fold-lower luciferase activities in CCR5-expressing HOS.CD4 cells but slightly higher values in cells expressing CCR1, CCR2B, and CCR3 compared to those of SIVmac316 (Fig. 3). Nonetheless, no major variations, like a switch from a form that uses predominantly CCR5 to a form that uses CXCR4, were observed.

A single change, 321P→S, in the SIVmac V3 loop impairs entry into CEMx174 cells. Pseudotyped SIVmac luciferase reporter viruses allow for quantitation of viral entry efficiency into susceptible cells. The T-B hybrid cell line CEMx174 is commonly used for SIVmac propagation and for virus reisolation from infected rhesus macaques. In agreement with the observation that SIVmac replicates with high efficiency in this hybrid cell line, high reporter gene activities were observed after infection (Fig. 4). Similar to the results obtained with CCR5-expressing HOS.CD4 cells, about 15- to 30-fold-higher luciferase values (23.4 ± 5.1; obtained in five independent experiments) were measured after infection with 316Env-containing virus compared to those for SIVmac239-pseudotyped

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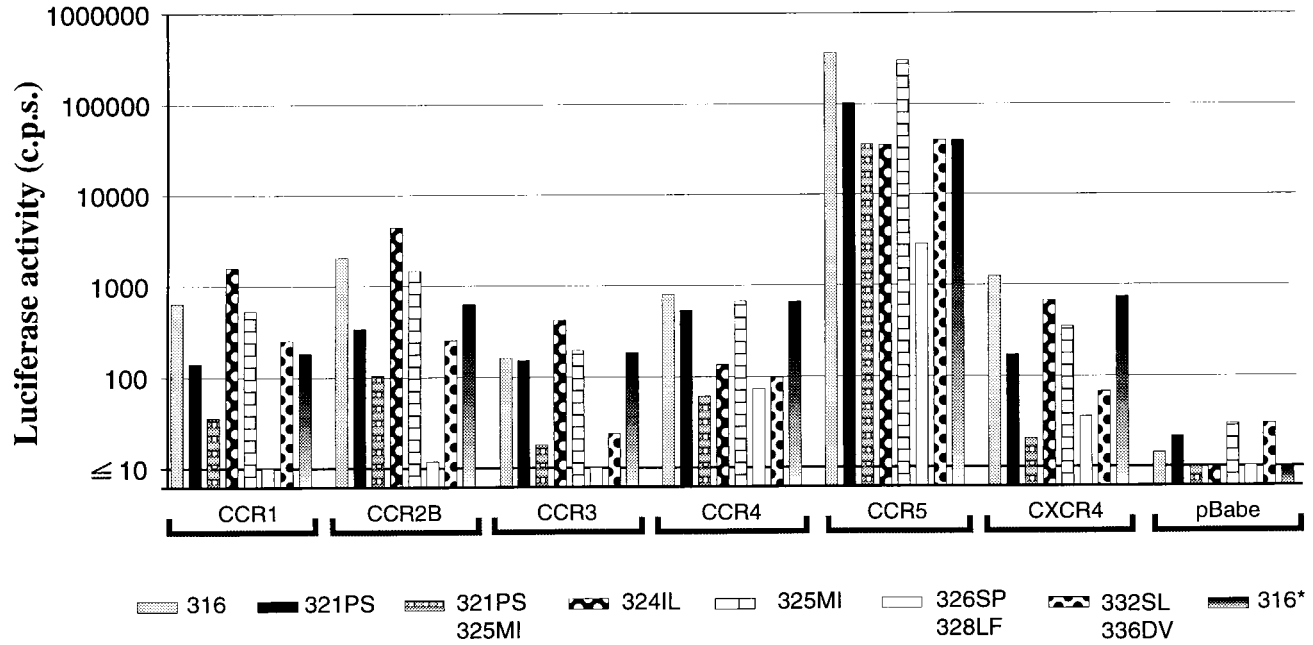

FIG. 3. Influence of changes in the V3 loop sequence of SIVmac239/316Env on entry into HOS.CD4 cells expressing various β-chemokine receptors. Cells were infected and tested for viral entry by luciferase assay as described in the legend to Fig. 2. Comparable results were obtained in two independent experiments.

particles. In contrast, only slightly higher levels of luciferase activities (3.1 ± 1.7) were observed after infection of C8166 cells with 316Env compared to those for 239Env-pseudotyped SIV particles (Fig. 4). It has previously been described that a single change, 321P→S, impairs viral replication in CEMx174 cells but has little influence on replication in macrophage and PBMC cultures (35). This variant used CCR5 for efficient entry (Fig. 3) and did enter into C8166 cells, although with reduced efficiency (Fig. 4). In contrast, the reporter gene activities obtained after infection of CEMx174 cells were only slightly above nonspecific background levels (Fig. 4A). A second variant containing 325M→I change in addition to the 321P→S substitution showed even lower entry efficiency in this cell line. Similar results were obtained when HIV-1 reporter virus pseudotyped with SIV Env variants was used (Fig. 4B). These results indicate that a single amino acid substitution in the SIVmac V3 loop, 321P→S, has high impact on the ability to use the coreceptor expressed in CEMx174 cells and much lower influence on fusions with other cofactors, like CCR5. In agreement with these results, reverse transcription-PCR anal-

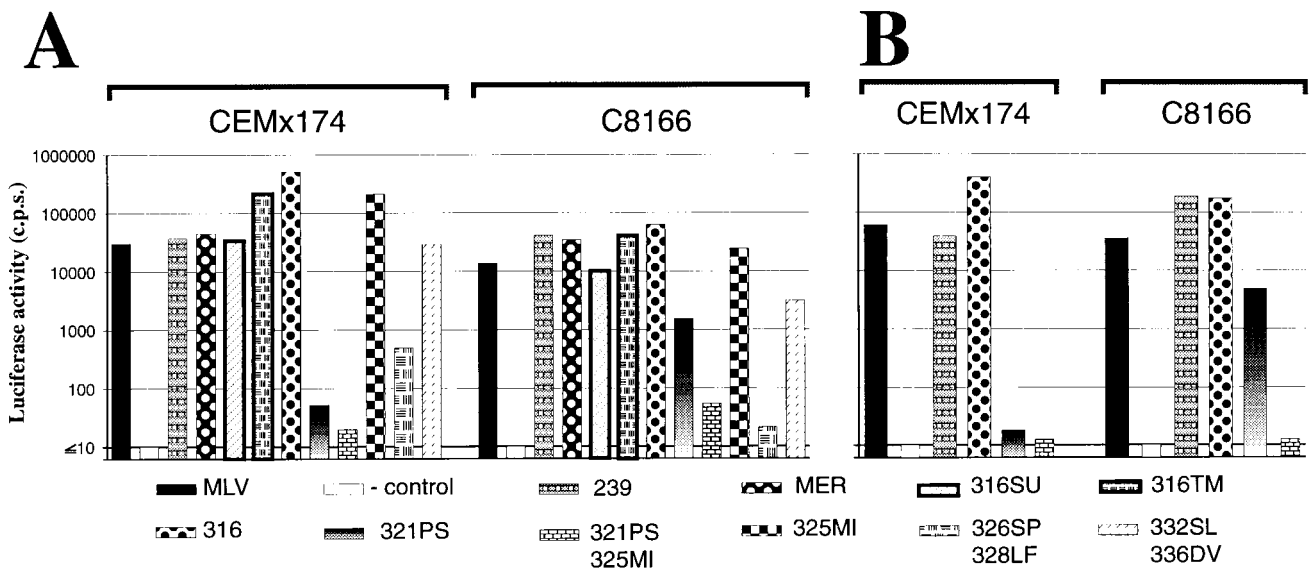

FIG. 4. Entry of pseudotyped SIV (A) and HIV (B) luciferase reporter viruses into CEMx174 and C8166 cells. Cells were diluted in fresh medium (1:2 [vol/vol]) and seeded out on 48-well plates the day before infection with virus containing 100 ng of p27 antigen in a total volume of 500 μl. After 16-h incubation, equal amounts of medium were added to all wells. After 4 days of culture, 50-μl extracts were prepared and the luciferase activity in 20 μl was assayed with a commercial kit (Promega). − control, only pBR239ΔenvLuc DNA transfected to generate viral particles.

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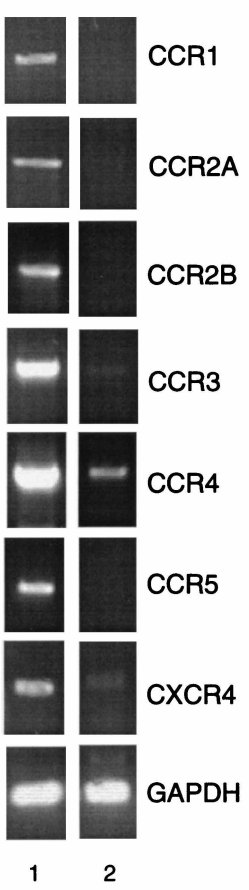

FIG. 5. Detection of β-chemokine receptor mRNAs in unstimulated human PBMC (lane 1) and CEMx174 cells (lane 2). RNA was prepared, reverse transcribed, and subjected to PCR analysis as described in Materials and Methods. PCR products were separated on 1% agarose gel. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

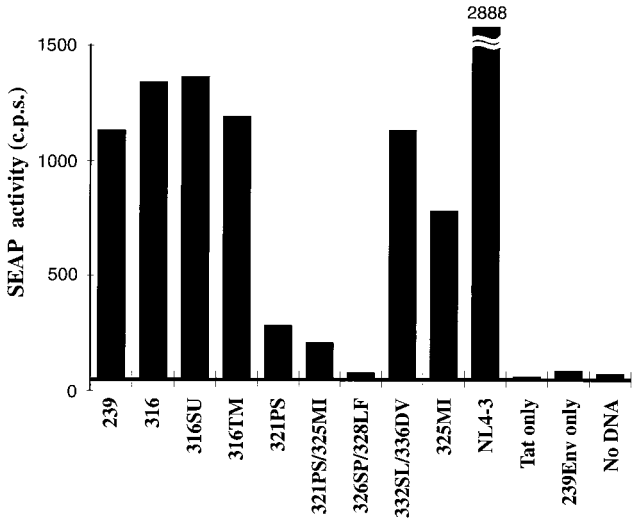

FIG. 6. Env-mediated cell-cell fusion. 293T cells were seeded on 24-well plates and cotransfected with 5 μg of pcDNA-SIVtat and 5 μg of pcDNA-Env expression plasmids on the following day as described in Materials and Methods. After overnight incubation, the medium was removed and replaced by 1 ml of RPMI 1640 medium containing 300,000 CEMx174-SEAP cells. Fusions of Env- and Tat-expressing 293T cells with CEMx174 cells led to activation of the gene encoding SEAP. The amount of SEAP released in the supernatant was assayed at 24 h after cocultivation.

ysis revealed that CEMx174 cells expressed relatively high levels of CCR4 and low levels of CCR3 and CXCR4, which are not major cofactors for SIVmac entry, but did not express CCR5 (Fig. 5). In contrast, mRNAs encoding CCR1, CCR2A, CCR2B, CCR3, CCR4, CCR5, and CXCR4 were readily detected in unstimulated human PBMC (Fig. 5). No significant entry into CEM cells by the SIVmac envelope variants analyzed in the present study was observed (data not shown).

Effects of SIVmac Env variations on cell-cell fusions. To assess the efficiencies of cell-cell fusions mediated by different SIVmac Env proteins, 293T cells were cotransfected with SIV Env and Tat expression plasmids and cocultivated with CEMx174 cells containing the SEAP reporter gene under the control of the SIV LTR. Fusions of transfected cells with CEMx174-SEAP cells led to activation of the SEAP gene and secretion of alkaline phosphatase into the medium. As shown in Fig. 6, significant amounts of SEAP activity were detected only after cocultivation of 293T cells expressing both Tat and Env. The 239, 316, 316SU, and 316TM envelopes showed comparable fusion activities in this assay. Similarly, V3 loop changes of 325M→I and 332S→L plus 336D→V had little influence on fusions. In contrast, the 321P→S substitution, which reduced the entry of pseudotyped particles about 10,000-fold (Fig. 4), resulted in about 10-fold-diminished cell-cell fusion activity. Although SIV reporter virus pseudotyped with the 326S→P and 328L→F mutant entered CEMx174 cells with higher efficiency compared to that of the 321P→S variant, the cell-cell fusion activities observed with this variant were close to nonspecific background levels (Fig. 6).

Entry into PBMC and macrophages. The observation that SIVmac239, SIVmac316, and the SIVmac316 321P→S mutant can use CCR5 for efficient entry suggests that these SIVmac variants can efficiently enter primary cells. As shown in Fig. 5, unstimulated human PBMC expressed a broad variety of β-chemokines, CCR1, CCR2A, CCR2B, CCR3, CCR4, CCR5, and CXCR4. 316Env mediated 5- to 10-fold-higher levels of luciferase reporter gene activities in both PBMC and macrophages of human and rhesus origins (Fig. 7A). Although SIVmac316 seemed to enter PBMC with 5- to 10-fold-higher efficiencies compared to those of SIVmac239, both forms replicated with comparable efficiencies in rhesus PBMC cultures (Fig. 7B). In contrast, in rhesus macrophage cultures, efficient viral replication was observed only after infection with SIVmac316. When the HIV-1 vector was used, no luciferase activities above background levels were observed in macaque cells (Fig. 7A).

DISCUSSION

For HIV-1, it has been demonstrated that macrophage-tropic strains use CCR5 as a potent coreceptor (1, 9, 17, 21) and that T-cell-tropic strains use predominantly CXCR4 (4, 22). In seeming contrast, we have found that both SIVmac239, which replicates very poorly in macrophages (39), and an envelope variant, SIVmac316, which replicates more efficiently (about 1,000 times) in macrophages can use CCR5, but not CXCR4, for efficient entry. However, SIVmac239 replication in macrophages does not seem to be blocked at the level of entry. Mori et al. found only about four-fold-higher levels of newly synthesized DNA in macrophages infected with SIVmac239/316Env compared to those with SIVmac239 (39). We observed about 5- to 10-fold-higher amounts of luciferase ac-

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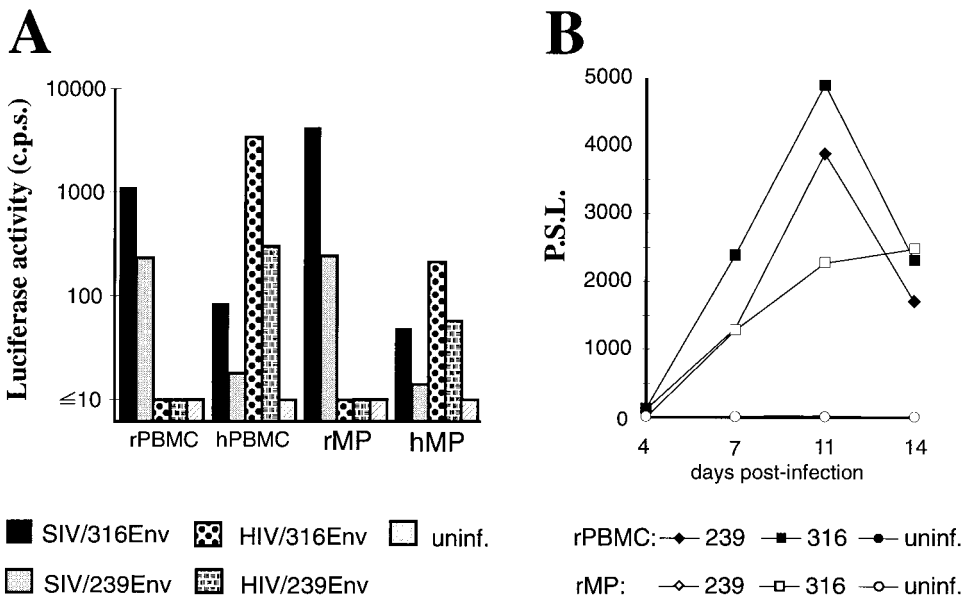

FIG. 7. Entries of 239Env- and 316Env-pseudotyped SIV and HIV strains into PBMC and macrophages (MP) of rhesus macaque (r) and human (h) origins. (A) Cells were isolated and cultured as described in Materials and Methods. Cells were infected with media containing pseudotyped SIV and HIV (100 ng of p27 and p24, respectively). Antigen concentrations and luciferase activities were measured at 4 days postinfection as described in Materials and Methods. (B). The cell preparations from panel A were used for infection with 5 ng of p27 antigen of either SIVmac239 or SIVmac316 virus. Reverse transcriptase activities were measured in parallel as previously described (46, 48). P.S.L., photo-stimulated luminescence; uninf., uninfected.

tivities in macrophage cultures after infection with 316Env-pseudotyped virus (Fig. 7). In contrast to the observations made with SIVmac239 in macrophages (detectable luciferase reporter activities, indicating efficient entry, reverse transcription, and integration), infection of several immortalized human cell lines (CEM, Jurkat, Molt-4, Molt 4 cl.8, SupT1, and U937) with pseudotyped reporter virus did not lead to significant levels of reporter gene activities (data not shown). Accordingly, SIVmac239 replication in macrophages and those human cell lines seems to be blocked at different stages; the mechanisms that restrict replication in macrophages remain to be elucidated.

None of the SIVmac envelope variants analyzed in this study used CXCR4 for efficient entry. The results do not necessarily mean, however, that the determinants of coreceptor and macrophage tropism are fundamentally different for SIVmac and HIV-1. Similar to SIVmac239, some HIV-1 isolates replicate poorly in macrophages, although they enter efficiently (52). Although the V3 loop is clearly an important determinant of HIV-1 macrophage tropism (6–8, 10, 25, 29, 41), some data also suggest that for HIV-1 these determinants can be complex (41, 53). Considering that none of the specific changes in Env that allow efficient replication of SIVmac316 in macrophages maps to V3 (39), it seems likely that the V3 loop sequences of SIVmac239 and SIVmac316 are close to optimum for efficient usage of CCR5 and entry into macrophages. Although the V3 loop of SIVmac seems to be conserved more often than is HIV-1 V3 (5, 30, 42), some V3 changes that are associated with enhanced cytopathicity and altered tissue tropism have previously been described (27, 33, 34). Moreover, analogous to HIV-1 infection in humans, the appearance of rapidly replicating, T-cell-tropic cytopathic SIVmac variants during the development of AIDS in infected macaques has previously been reported (49). It will be interesting to examine whether any of the envelope changes described here is associated with efficient usage of CXCR4.

One of the most intriguing results of this study is that CEMx174 cells, which are highly susceptible to infection with SIVmac and show high levels of viral entry, do not express CCR5. A single 321P→S substitution in the 316 V3 loop sequence almost completely impaired viral entry into CEMx174 cells but had much less impact on entry into CCR5-expressing HOS.CD4 cells and C8166 cells. Previously, it has been reported that this change drastically reduces viral replication in CEMx174 cells but not in rhesus PBMC or macrophages (32). Thus, analogous to HIV-1 V3, single amino acid substitutions in SIVmac V3 can have differential effects on the ability to use various coreceptors for efficient entry. Since no entry into the parental CEM cell line was observed (data not shown), the hybrid cells seem to express a currently unidentified, perhaps B-cell-derived cofactor.

CCR5 seems to play a central role in the transmission of HIV-1 and in viral replication, at least at the early stages of infection before cytopathic T-cell-tropic variants occur (1, 9, 16, 17, 21, 28, 36, 45, 51). Our data show that similar to primary HIV-1 isolates, SIVmac uses CCR5 for efficient entry and that the V3 loop of SIVmac can affect coreceptor usage in a way analogous to that of HIV-1 V3. Therefore, experimental infection of rhesus macaques with SIVmac may provide a useful model for studying the relevance of inhibitory chemokines for the prevention of infection and for therapeutic approaches against AIDS. Furthermore, we show that CEMx174 cells seem to express an as-yet-unidentified coreceptor. Further studies are required to investigate the role(s) of these additional cofactors in SIV and HIV entry and pathogenesis.

ACKNOWLEDGMENTS

F. Kirchhoff and S. Pöhlmann contributed equally to this work.

We thank Marianne Haupt for excellent technical assistance, Nathaniel R. Landau for HOS.CD4 cells stably expressing β-chemokine receptors and the NL4-3-Luc-R$^-$E$^-$ clone, and Ronald C. Des-

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rosiers, Kazuyasu Mori, and Toshiaki Kodama for envelope plasmids and SIVmac clones. We also thank Bernhard Fleckenstein for support.

This work was supported by Sonderforschungsbereich 466 and a fellowship from the German BMBF to F.K.

REFERENCES

1. **Alkhatib, G., C. Combadiere, C. C. Broder, Y. Feng, P. E. Kennedy, P. M. Murphy, and E. A. Berger.** 1996. CC-CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1. Science **272:**1955–1958.
2. **Ashorn, P. A., E. A. Berger, and B. Moss.** 1990. Human immunodeficiency virus envelope glycoprotein/CD4-mediated fusion of nonprimate cells with human cells. J. Virol. **64:**2149–2156.
3. **Banapour, B., M. L. Marthas, R. A. Ramos, B. L. Lohman, R. E. Unger, M. B. Gardner, N. C. Pedersen, and P. A. Luciw.** 1991. Identification of viral determinants of macrophage tropism for simian immunodeficiency virus SIVmac. J. Virol. **65:**5798–5805.
4. **Berson, J. F., D. Long, B. J. Doranz, J. Rucker, F. R. Jirik, and R. W. Doms.** 1996. A seven-transmembrane domain receptor involved in fusion and entry of T-cell-tropic human immunodeficiency virus type 1 strains. J. Virol. **70:** 6288–6295.
5. **Burns, D. P., and R. C. Desrosiers.** 1991. Selection of genetic variants of simian immunodeficiency virus in persistently infected rhesus monkeys. J. Virol. **65:**1843–1854.
6. **Cann, A. J., M. J. Churcher, M. Boyd, W. O'Brien, J.-Q. Zhao, J. Zack, and I. S. Y. Chen.** 1992. The region of the envelope gene of human immunodeficiency virus type 1 responsible for determination of cell tropism. J. Virol. **66:**305–309.
7. **Cheng-Mayer, C., M. Quiroga, J. W. Tung, D. Dina, and J. A. Levy.** 1990. Viral determinants of human immunodeficiency virus type 1 T-cell or macrophage tropism, cytopathogenicity, and CD4 antigen modulation. J. Virol. **64:**4390–4398.
8. **Chesebro, B., K. Wehrly, J. Nishio, and S. Perryman.** 1992. Macrophage-tropic human immunodeficiency virus isolates from different patients exhibit unusual V3 envelope sequence homogeneity in comparison with T-cell-tropic isolates: definition of critical amino acids involved in cell tropism. J. Virol. **66:**6547–6554.
9. **Choe, H., M. Farzan, Y. Sun, N. Sullivan, B. Rollins, P. D. Ponath, L. Wu, C. R. Mackay, G. LaRosa, W. Newman, N. Gerard, C. Gerard, and J. Sodroski.** 1996. The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. Cell **85:**1135–1148.
10. **Cocchi, F., A. L. DeVico, A. Garzino-Demo, A. Cara, R. C. Gallo, and P. Lusso.** 1996. The V3 domain of the HIV-1 gp120 envelope glycoprotein is critical for chemokine-mediated blockage of infection. Nat. Med. **2:**1244–1247.
11. **Collman, R., B. Godfrey, J. Cutilli, A. Rhodes, N. F. Hassan, R. Sweet, S. D. Douglas, H. Friedman, N. Nathanson, and F. Gonzalez-Scarano.** 1990. Macrophage-tropic strains of human immunodeficiency virus type 1 utilize the CD4 receptor. J. Virol. **64:**4468–4476.
12. **Combadiere, C., S. K. Ahuja, H. L. Tiffany, and P. M. Murphy.** 1996. Cloning and functional expression of CC CKR5, a human monocyte CC chemokine receptor selective for MIP-1(alpha), MIP-1(beta), and RANTES. J. Leukocyte Biol. **60:**147–152.
13. **Connor, R. I., B. K. Chen, S. Choe, and N. R. Landau.** 1995. Vpr is required for efficient replication of human immunodeficiency virus type-1 in mononuclear phagocytes. Virology **206:**935–944.
14. **Cordonnier, A., L. Montagnier, and M. Emerman.** 1992. Single amino-acid changes in HIV envelope affect viral tropism and receptor binding. Nature (London) **340:**571–574.
15. **Dalgleish, A. G., P. C. L. Beverley, P. R. Clapham, D. H. Crawford, M. F. Greaves, and R. A. Weiss.** 1984. The CD4 (T4) antigen is an essential component of the receptor for the AIDS virus. Nature (London) **312:**763–767.
16. **Dean, M., M. Carrington, C. Winkler, G. A. Huttley, M. W. Smith, R. Allikmets, J. J. Goedert, S. P. Buchbinder, E. Vittinghoff, E. Gomperts, S. Donfield, D. Vlahov, R. Kaslow, A. Saah, C. Rinaldo, R. Detels, and S. J. O'Brien.** 1996. Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Science **273:**1856–1862.
17. **Deng, H., R. Liu, W. Ellmeier, S. Choe, D. Unutmaz, M. Burkhart, P. Di Marzio, S. Marmon, R. E. Sutton, C. M. Hill, C. B. Davis, S. C. Peiper, T. J. Schall, D. R. Littman, and N. R. Landau.** 1996. Identification of a major co-receptor for primary isolates of HIV-1. Nature (London) **381:**661–666.
18. **Desrosiers, R. C.** 1990. The simian immunodeficiency viruses. Annu. Rev. Immunol. **8:**557–578.
19. **Desrosiers, R. C., A. Hansen-Moosa, K. Mori, D. P. Bouvier, N. W. King, M. D. Daniel, and D. J. Ringler.** 1991. Macrophage-tropic variants of SIV are associated with specific AIDS-related lesions but are not essential for the development of AIDS. Am. J. Pathol. **139:**29–35.
20. **Doranz, B. J., J. Rucker, Y. Yi, R. J. Smyth, M. Samson, S. C. Peiper, M. Parmentier, R. G. Collman, and R. W. Doms.** 1996. A dual-tropic primary HIV-1 isolate that uses fusin and the beta-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. Cell **85:**1149–1158.
21. **Dragic, T., V. Litwin, G. P. Allaway, S. R. Martin, Y. Huang, K. A. Nagashima, C. Cayanan, P. J. Maddon, R. A. Koup, J. P. Moore, and W. A. Paxton.** 1996. HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. Nature (London) **381:**667–673.
22. **Feng, Y., C. C. Broder, P. E. Kennedy, and E. A. Berger.** 1996. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. Science **272:**872–877.
23. **Gardner, M. B., and P. A. Luciw.** 1989. Animal models of AIDS. FASEB J. **3:**2593–2606.
24. **Gartner, S., P. Markovits, D. M. Markovitz, M. H. Kaplan, R. C. Gallo, and M. Popovic.** 1986. The role of mononuclear phagocytes in HTLV-III/LAV infection. Science **233:**215–219.
25. **Grimaila, R. J., B. A. Fuller, P. D. Rennert, M. B. Nelson, M. L. Hammarskjold, B. Potts, M. Murray, S. D. Putney, and G. Gray.** 1992. Mutations in the principal neutralization determinant of human immunodeficiency virus type 1 affect syncytium formation, virus infectivity, growth kinetics, and neutralization. J. Virol. **66:**1875–1883.
26. **Gundlach, B. R., H. Linhart, U. Dittmer, S. Sopper, S. Reiprich, D. Fuchs, B. Fleckenstein, G. Hunsmann, C. Stahl-Hennig, and K. Überla.** 1997. Construction, replication, and immunogenic properties of a simian immunodeficiency virus expressing interleukin 2. J. Virol. **71:**2225–2232.
27. **Hirsch, V. M., J. E. Martin, G. Dapolito, W. R. Elkins, W. T. London, S. Goldstein, and P. R. Johnson.** 1994. Spontaneous substitutions in the vicinity of the V3 analog affect cell tropism and pathogenicity of simian immunodeficiency virus. J. Virol. **68:**2649–2661.
28. **Huang, Y., W. A. Paxton, S. M. Wolinsky, A. U. Neumann, L. Zhang, T. He, S. Kang, D. Ceradini, Z. Jin, K. Yazdanbakhsh, K. Kunstman, D. Erickson, E. Dragon, N. R. Landau, J. Phair, D. D. Ho, and R. A. Koup.** 1996. The role of a mutant CCR5 allele in HIV-1 transmission and disease progression. Nat. Med. **2:**1240–1243.
29. **Hwang, S. S., T. J. Boyle, H. K. Lyerly, and B. R. Cullen.** 1991. Identification of the envelope V3 loop as the primary determinant of macrophage tropism in HIV-1. Science **253:**71–74.
30. **Johnson, P. R., T. E. Hamm, S. Goldstein, S. Kitov, and V. M. Hirsch.** 1991. The genetic fate of molecularly cloned simian immunodeficiency virus in experimentally infected macaques. Virology **185:**217–228.
31. **Kestler, H. W., T. Kodama, D. Ringler, M. Marthas, N. Pedersen, A. Lackner, D. Regier, P. Sehgal, M. D. Daniel, and R. C. Desrosiers.** 1990. Induction of AIDS in rhesus monkeys by molecularly cloned simian immunodeficiency virus. Science **248:**1109–1112.
32. **Kirchhoff, F., K. Mori, and R. C. Desrosiers.** 1994. The "V3" domain is a determinant of simian immunodeficiency virus cell tropism. J. Virol. **68:** 3682–3692.
33. **Kodama, T., K. Mori, T. Kawahara, D. J. Ringler, and R. C. Desrosiers.** 1993. Analysis of simian immunodeficiency virus sequence variation in tissues of rhesus macaques with simian AIDS. J. Virol. **67:**6522–6534.
34. **LaBranche, C. C., M. M. Sauter, B. S. Haggarty, P. J. Vance, J. Romano, T. K. Hart, P. J. Bugelski, and J. A. Hoxie.** 1994. Biological, molecular, and structural analysis of a cytopathic variant from a molecularly cloned simian immunodeficiency virus. J. Virol. **68:**5509–5522.
35. **Letvin, N. L., M. D. Daniel, P. K. Sehgal, R. C. Desrosiers, R. D. Hunt, L. M. Waldron, J. J. MacKey, D. K. Schmidt, L. V. Chalifoux, and N. W. King.** 1985. Induction of AIDS-like disease in macaque monkeys with T-cell tropic retrovirus STLV-III. Science **230:**71–73.
36. **Liu, R., W. A. Paxton, S. Choe, D. Ceradini, S. R. Martin, R. Horuk, M. E. MacDonald, H. Stuhlmann, R. A. Koup, and N. R. Landau.** 1996. Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. Cell **86:**367–377.
37. **Maddon, P. J., A. G. Dalgleish, J. S. McDougal, P. R. Clapham, R. A. Weiss, and R. Axel.** 1986. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. Cell **47:**333–348.
38. **Means, R. E., T. Greenough, and R. C. Desrosiers.** Neutralization sensitivity of cell culture-passaged simian immunodeficiency virus. J. Virol., in press.
39. **Mori, K., D. J. Ringler, T. Kodama, and R. C. Desrosiers.** 1992. Complex determinants of macrophage tropism in *env* of simian immunodeficiency virus. J. Virol. **66:**2067–2075.
40. **Mori, K., D. J. Ringler, and R. C. Desrosiers.** 1993. Restricted replication of simian immunodeficiency virus strain 239 in macrophages is determined by env but is not due to restricted entry. J. Virol. **67:**2807–2814.
41. **O'Brien, W. A., Y. Koyanagi, A. Namazie, J.-Q. Zhao, A. Diagne, K. Idler, J. A. Zack, and I. S. Y. Chen.** 1990. HIV-1 tropism for mononuclear phagocytes can be determined by regions of gp120 outside the CD4 binding domain. Nature (London) **348:**69–73.
42. **Overbaugh, J., L. M. Rudensey, M. D. Papenhausen, R. E. Benveniste, and W. R. Morton.** 1991. Variation in simian immunodeficiency virus env is confined to V1 and V4 during progression to simian AIDS. J. Virol. **65:** 7025–7031.
43. **Page, K. A., N. R. Landau, and D. R. Littman.** 1990. Construction and use of a human immunodeficiency virus vector for analysis of virus infectivity. J. Virol. **64:**5270–5276.
44. **Page, K. A., S. M. Stearns, and D. R. Littman.** 1992. Analysis of mutations

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in the V3 domain of gp160 that affect fusion and infectivity. J. Virol. **66:**524–533.
45. **Paxton, W. A., S. R. Martin, D. Tse, T. R. O'Brien, J. Skurnick, N. L. VanDevanter, N. Padian, J. F. Braun, D. P. Kotler, S. M. Wolinsky, and R. A. Koup.** 1996. Relative resistance to HIV-1 infection of CD4 lymphocytes from persons who remain uninfected despite multiple high-risk sexual exposures. Nat. Med. **2:**412–417.
46. **Potts, B. J.** 1990. "Mini" reverse transcriptase (RT) assay, p. 103–106. *In* A. Aldovini and B. D. Walker (ed.), Techniques in HIV research. Stockton, New York, N.Y.
47. **Regier, D. A., and R. C. Desrosiers.** 1990. The complete nucleotide sequence of a pathogenic molecular clone of SIV. AIDS Res. Hum. Retroviruses **6:**1221–1231.
48. **Reiprich, S., R. B. Gundlach, B. Fleckenstein, and K. Überla.** 1997. Replication-competent chimeric lenti-oncovirus with expanded host cell tropism. J. Virol. **71:**3328–3331.
49. **Rudensey, L. M., J. T. Kimata, R. E. Benveniste, and J. Overbaugh.** 1995. Progression to AIDS in macaques is associated with changes in the replication, tropism, and cytopathic properties of the simian immunodeficiency virus variant population. Virology **207:**528–542.
50. **Samson, M., O. Labbe, C. Mollereau, G. Vassart, and M. Parmentier.** 1996. Molecular cloning and functional expression of a new human CC-chemokine receptor gene. Biochemistry **19:**3362–3367.
51. **Samson, M., F. Libert, B. J. Doranz, J. Rucker, C. Liesnard, C. M. Farber, S. Saragosti, C. Lapoumeroulie, J. Cognaux, C. Forceille, G. Muyldermans, C. Verhofstede, G. Burtonboy, M. Georges, T. Imai, S. Rana, Y. Yi, R. J. Smyth, R. G. Collman, R. W. Doms, G. Vassart, and M. Parmentier.** 1996. Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. Nature (London) **382:**722–725.
52. **Schmidtmayerova, H., C. Bolmont, S. Baghdiguian, I. Hirsch, and J. C. Chermann.** 1992. Distinctive pattern of infection and replication of HIV-1 strains in blood-derived macrophages. Virology **190:**124–133.
53. **Shioda, T., J. A. Levy, and C. Cheng-Mayer.** 1991. Macrophage and T-cell tropisms of HIV-1 are determined by specific regions of the envelope gp120 gene. Nature (London) **349:**167–169.

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