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Generation of Intersubtype Human Immunodeficiency Virus Type 1 Recombinants in *env* Gene *In Vitro*: Influences in the Biological Behavior and in the Establishment of Productive Infections

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The occurrence of human immunodeficiency virus type 1 (HIV-1) recombinant genomes belonging to different subtypes is a common event in regions where more than two subtypes cocirculate. Although there are accumulating data toward an increase in the number of intersubtype recombinants, little has been addressed about the biological behavior of such mosaic genomes. This work reports the biological characterization of engineered *in vitro* HIV-1 intersubtype recombinants in the gp120 region. The recombinants possess the entire gp120 of B or F Brazilian isolates in the Z6 (subtype D) backbone. Here we show that this type of recombinant structure results in profound impairment to the establishment of productive infections in CD4-positive cells. The characterization of biological properties of those recombinant viruses demonstrated viral production occurring only during a transient peak early on infection and that they are not able to down-regulate the expression of CD4 receptor on the cell surface. We also report the phenotype reversion of one recombinant virus studied here, after 62 days in culture. Two amino acid substitutions in highly constant gp120 regions (C1 and C4) were identified in the revertant virus. The mutation occurring in the C4 region is localized near two amino acid residues critical for gp120/CD4 interaction. Based on these data, we suggest that failure in CD4 down-modulation by recombinant viruses can be due to a structural dysfunction of gp160 protein unable to block CD4 at the endoplasmic reticule. The possibilities that the establishment of latent infections can be directly related to the continuous expression of CD4 on the infected cell surface and that the occurrence of mutations in amino acid nearby residues critical for gp120/CD4 interaction can restore the fully productive infectious process are discussed.

INTRODUCTION

Among all of the characteristics of human immunodeficiency virus type 1 (HIV-1), the most extensively studied is its growing genetic variability. Nucleotide sequence analyses of *gag* and *env* viral genes, obtained from infected patients worldwide, led to a classification system of these samples based on their phylogenetic relationships (McCutchan *et al.,* 1992; Myers *et al.,* 1995). HIV-1 strains globally can be classified at least in 10 different subtypes (A–J) of the main (M) group. A number of sequences exist that differ in more than 50% from the sequences of the M group and from each other, and they are classified into the group O ("outliner") (Korber *et al.,* 1997). Finally, highly divergent sequences distinct from both M and O groups have been classified as group N (Simon *et al.,* 1998). The relatively low fidelity of viral reverse transcriptase (Mansky and Temin, 1995) combined with the extremely high turnover of virus *in vivo* (10^9 virions/day) provides the basis for the continuous emergence of new HIV variants (Ho *et al.,* 1995; Wei *et al.,* 1995).

Like all retroviruses, each HIV-1 viral particle contains two RNA strands of positive polarity. Typically both RNA strands in a retroviral particle are derived from the same parent provirus (Artenstein *et al.,* 1995). However, if an infected cell simultaneously harbors two different proviruses, one RNA transcript from each provirus can be encapsidated into a single "heterozygous" virion. For the reverse transcription (RT) process, due to the characteristics of the virus reverse transcriptase, this enzyme may jump back and forth between the two RNA templates, and as a result the newly synthesized retroviral DNA sequence is a recombinant between the two parental strands (Hu and Temin, 1990a,b). In fact, interclade HIV-1 recombinants have been commonly detected in geographic regions where two or more clades are prevalent (Louwagie *et al.,* 1995). Recombination contributes to the generation of genetic diversity in HIV-1, and intersubtype recombinant sequence accounts for more than 10% of all isolates identified (Burke, 1997; Robertson *et al.,* 1995a,b). However, not all possible combinations between HIV-1 strains may give rise to recombinants in

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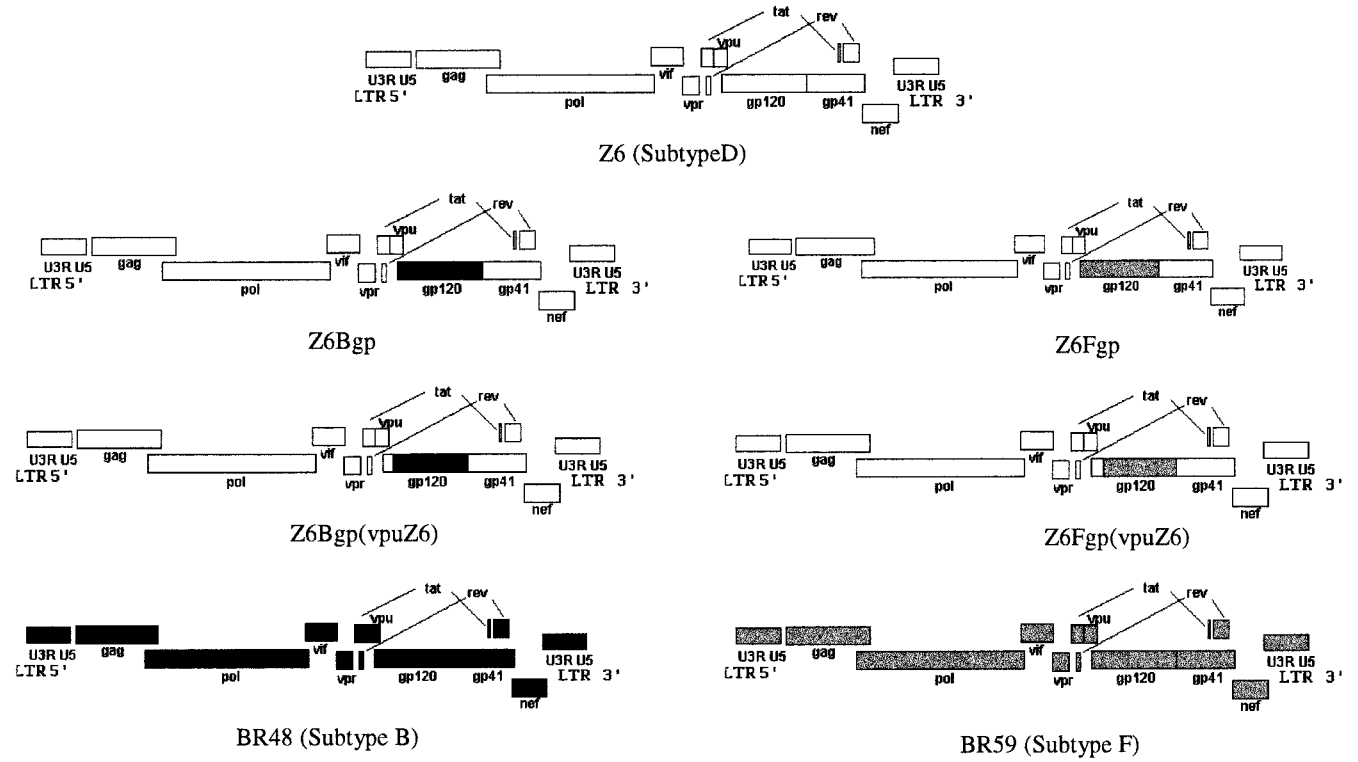

FIG. 1. Schematic representation of recombinant [pZ6Bgp, pZ6Bgp(vpuZ6), pZ6Fgp and pZ6Fgp(vpuZ6)] and parental (pZ6, BR48, BR59) virus genomes.

nature, mostly because of viral structural incompatibilities in intermolecular protein interaction (Burke, 1997).

The objective of this work was to study engineered intersubtype recombinants of HIV-1 in the *env* gene to access their biological properties and to replicate behavior in comparison with their highly replication competent parental viruses. For this purpose, we manipulated an HIV-1 infectious clone belonging to subtype D by swapping the *env* gene region by the same genomic region from two Brazilian isolates belonging to B and F subtypes. The biological properties and potential of the recombinant viruses to establish productive infections in HIV-1 permissive cells were evaluated.

RESULTS

Recombinant plasmid characterization and cell transfections

The replacement of pZ6 gp120 for the same region of subtype B or F viral isolates was confirmed by restriction map analysis of each construct and by dideoxynucleotide sequencing (Fig. 1). Both junction boundaries between the polymerase chain reaction (PCR) products and the entire gp120 B or F gene were identical to those expected for the procedure, and the nucleotide integrity of each construct was observed (data not shown). Established cultures of HeLa cells were transfected with those recombinant plasmids and with pZ6 used as control. We observed the same kinetics of viral particle production for all of the recombinants and the parental infectious clones (Fig. 2A), indicating the correct assembly of recombinant virions. Moreover, titration of both parental and recombinant viruses yielded the same amount of viral particles when assayed by infectious foci using MAGI cells (data not shown). We then decided to characterize the viral protein pattern produced in HeLa-transfected cells. For this purpose, viral particles released in the supernatant of metabolic ^{35}S-labeled HeLa cultures were purified by a sucrose cushion and treated with lysis buffer. The viral proteins were immunoprecipitated with antibody anti-gp120 or anti-HIV-1 (Fig. 2B). As could be observed, HIV-1 gp120 and gp41 from all viral constructs were immunoprecipitated when anti-HIV serum was used (Fig. 2B, lanes 3–5). Interestingly, only gp120 was immunoprecipitated in the chimerical constructs when the anti-gp120 antibody was used (Fig. 2B, lanes 6 and 7), suggesting a weaker interaction between gp120 and gp41 in these virions compared with that of Z6 (Fig. 2B, lane 8).

Chimeric virus infections in susceptible cells

Viruses harvested from HeLa cell supernatants were used to infect primary and established lymphocyte cultures. Phytohemagglutinin (PHA)-stimulated, interleukin (IL)-2 activated peripheral blood mononuclear cells (PBMCs) were

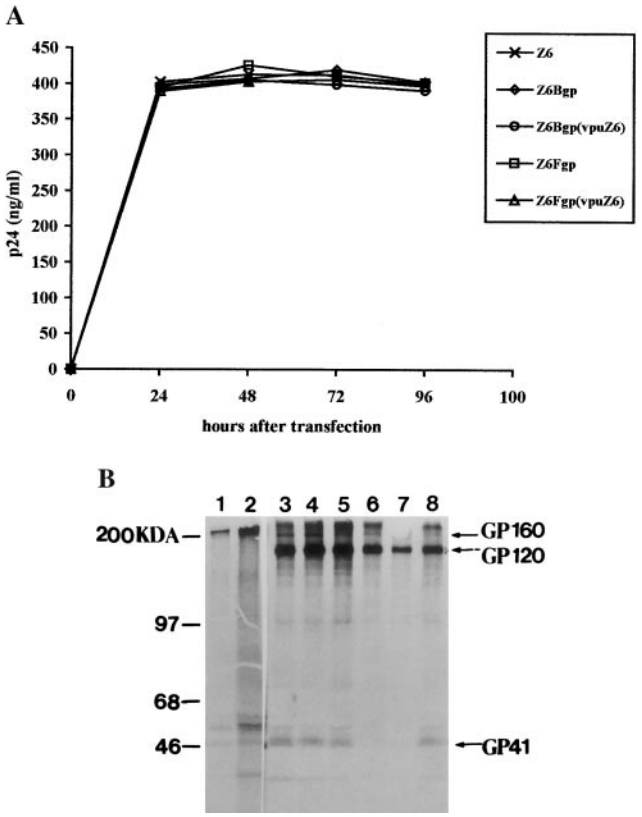

FIG. 2. Transfection of HeLa cells with infectious clones and radioimmunoprecipitation of HIV-1 glycoproteins from supernatants of transfected cells. (A) Monolayers of HeLa cells were transfected with 1 μg of each plasmid according to the LipofectAMINE protocol (GIBCO BRL). Several hours after transfection, aliquots of cell supernatants were collected and tested for the presence of p24 viral antigen using a specific ELISA assay (Coulter-Krefeld, Germany). (B) Transfected cultures were metabolic labeled with ^{35}S-methionine, and the viral particles released at the supernatant were purified by a sucrose cushion. The purified viruses were treated with lysis buffer, and the viral proteins were immunoprecipitated with anti-gp120 antibodies or anti-HIV-1 serum. The figure represents the autoradiography of a 12.5% SDS–polyacrylamide gel. Lanes 1 and 2, anti-gp120 or anti-HIV-1 immunoprecipitation of nontransfected cells, respectively. Lanes 3–5, anti-HIV-1 immunoprecipitation of Z6Bgp, Z6Fgp, and Z6 from the supernatant of transfected cells. Lanes 6–8, anti-gp120 immunoprecipitation of Z6Bgp, Z6Fgp, and Z6 from the supernatant of transfected cells. The molecular weight of proteins used as markers is indicated on the left.

infected with an m.o.i. of 0.1, and the kinetics of virus production was monitored every 24 h up to a 1-week period (Fig. 3). As could be observed, PBMCs infected with B/D or F/D viral chimeras reached a peak of virus production within 72 and 48 h after infection, respectively. However, viral production dropped after the transient peak, and after 7 days, viral particles were barely observed in the supernatants. Moreover, we noticed that Z6Fgp and Z6Fgp(vpuZ6) infections, which had reduced virion levels since the first 24 h after infection, were more severely impaired than those of Z6Bgp and Z6Bgp(vpuZ6).

The inability of recombinant viruses to sustain virus production was not due to interference in virus binding or

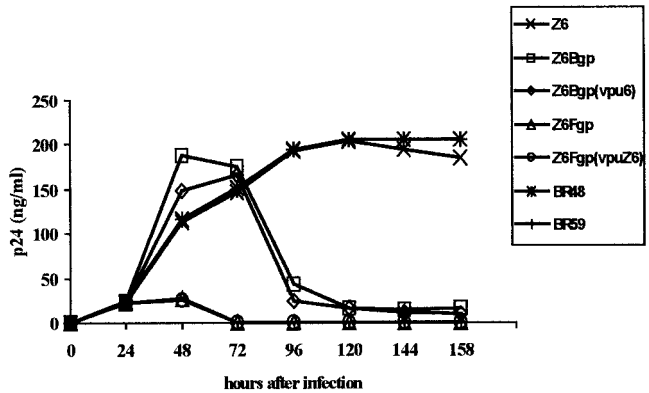

FIG. 3. Infection of susceptible CD4$^+$ cells with parental and recombinant viruses. Viruses harvested from HeLa cell supernatants were used to infect primary and established lymphocyte CD4$^+$ cultures. PHA-stimulated, IL-2-activated PBMCs were infected with an m.o.i. of 0.1, and the kinetics of viral production was monitored each 24 h up to a 1-week period using an ELISA-p24 assay. The data are representative of three independent experiments.

blocking in virus entry into target cells as it was observed in the p24 uptake assays (Aiken and Trono, 1995). Normalized amounts of recombinant or parental viruses were incubated with CEM cells at either 4°C or 37°C. The p24 uptake in assays carried out at 4°C has demonstrated that recombinant viruses attached to target cells as efficiently as parental viruses (Fig. 4). We could also observe, in assays conducted at 37°C, that the entry process of recombinant viruses was not disturbed and was quantitatively equivalent to the parental one (Fig. 4).

Although cultures infected by recombinant viruses could not establish productive infections, they exhibited an extensive formation of large syncytia in CEMss cultures during the first 7 days of infection (data not shown). These cells were maintained in culture for up to 14 days.

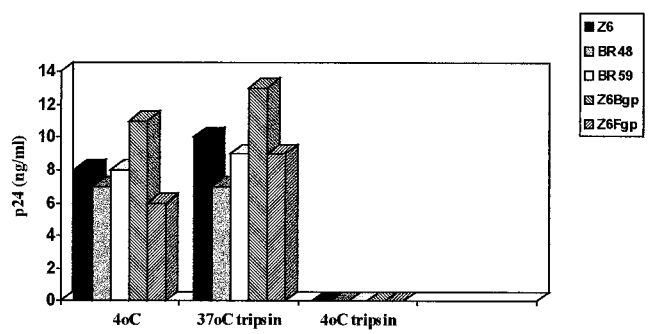

FIG. 4. Evaluation of viral entry into CD4$^+$ CEMss cells. Normalized amounts of recombinant (Z6Bgp or Z6Fgp) or parental (Z6, BR48, or BR59) viruses were incubated with CEMss cells at either 4°C or 37°C for 3 h. After an extensive washing, the amount of viral particles attached to the cell surface (4°C incubation) or inside cell cytoplasm (37°C incubation) was tested using an ELISA-p24 assay. Cells incubated with viruses at 37°C were treated with tripsin before p24 uptake evaluation to remove the particles simply attached to the cell surface. The data are representative of three independent experiments.

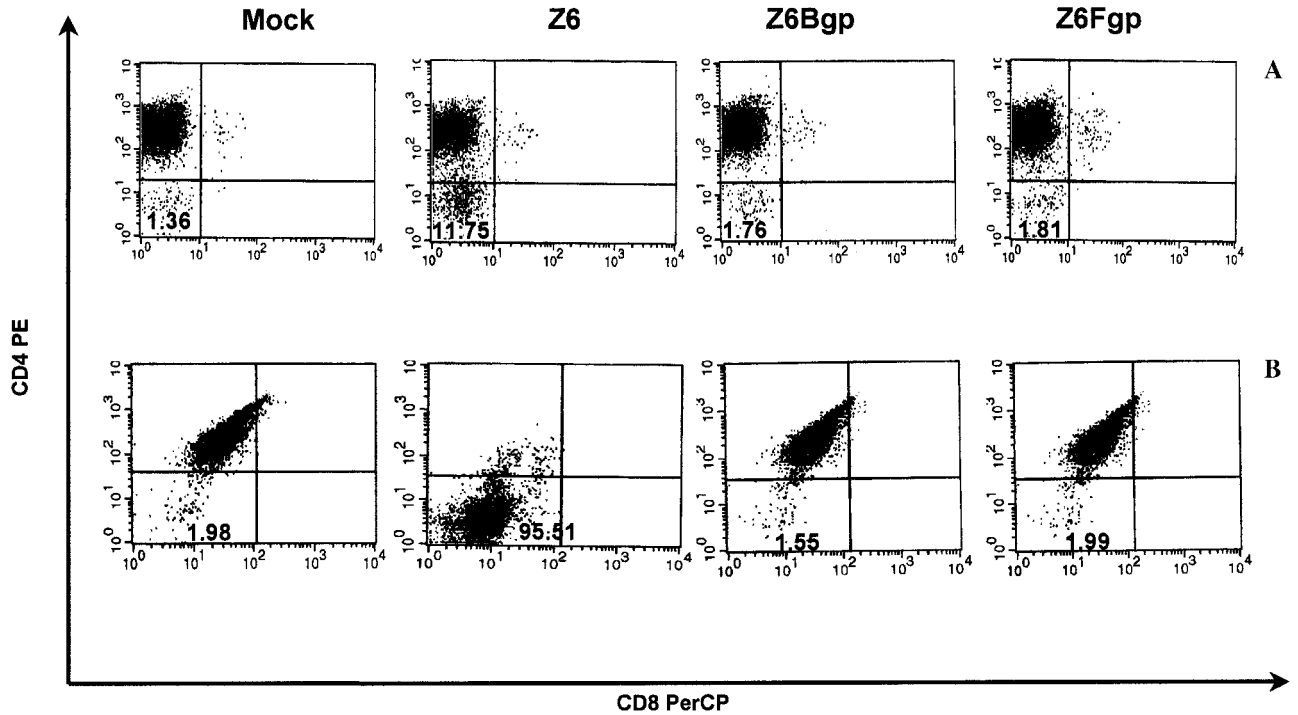

FIG. 5. Expression of CD4 molecules at the surface of CEMss cells. Cells infected with parental (Z6) or recombinant (Z6Bgp or Z6Fgp) viruses or mock infected were double incubated with FITC-conjugated anti-CD4 and anti-CD8 antibodies at 7 (A) and 14 (B) days after infection. The amount of CD4 at the cell membranes was determined through analyses in an FACSscan (Becton Dickinson) with CELLQuest software. The values shown at the lower left quadrant express the percentage of CD4-negative cells in the total amount of cells.

Although a great number of large syncytia could be observed in cultures infected with the parental viruses, in those infected with recombinant viruses syncytia were rarely seen (data not shown). We have performed tests of cell viability in PBMCs and CEMss cultures after 7 and 14 days of infection, and we could notice that recombinant and parental viruses killed the same amount of cells after a 7-day period. However, after 14 days of infection, only 25% of cells remained viable in parental virus-infected cultures, whereas in recombinant virus-infected cultures, viable cells represented 75% of the total amount (data not shown). Total amounts of viral DNA inside the infected cells were determined with quantitative PCR. Using this technique, we were able to detect the same amount of viral DNA in cell cultures infected with the recombinant viruses in all time points (from 24 h up to 14 days)after infection as we had detected for the Z6-infected cultures (data not shown).

Because the viral glycoprotein precursor (gp160) is responsible for the down-regulation of CD4 molecules at the surface of HIV-1-infected cells (Bour *et al.,* 1995), the next step was to analyze, 7 and 14 days after infection, the expression of the CD4 molecules at the surface of parental or recombinant virus-infected and mock-infected CEMss cells. We could observe that cells infected with parental viruses progressed to a complete inhibition of the expression of this molecule in the cell surface as expected for the HIV-1 natural infection course (Bour *et al.,* 1995). On the other hand, cells infected with recombinant viruses behaved as the mock-infected culture, expressing the same amounts of CD4 molecule in their surfaces during the entire period of infection with no signs of CD4 down-modulation (Figs. 5A and 5B).

Z6/Z6Fgp coinfection

We have tested the hypothesis that recombinant virus infection inefficiency might be due to a commitment in intercellular functions of chimerical gp160. In an attempt to revert this phenotype, PBMC cultures were coinfected with Z6 and Z6Fgp viruses at the same time. Fresh PBMCs were simultaneously challenged by a mixture containing approximately 1:10 Z6/Z6Fgp viral particles in a total m.o.i. of 0.1. We noticed in the coinfected culture a continuous production of viral particles at high levels throughout the experiment (Fig. 6A). At days 4, 8, 12, 15, and 18 after infection, the viruses released in the culture supernatant were harvested and genetically analyzed. To identify all viruses present, the gp120 gene was RT-PCR amplified, and the final products were submitted to *Eco*RI digestion. Because gp120 sequence from Z6 virus has a restriction site for this endonuclease that is absent in the gp120 sequence from Z6Fgp virus, we could distinguish between the two sequences. Figure 6B shows that at the beginning of coinfection, we could only identify Z6 virus at the supernatant. As the infection proceeded and

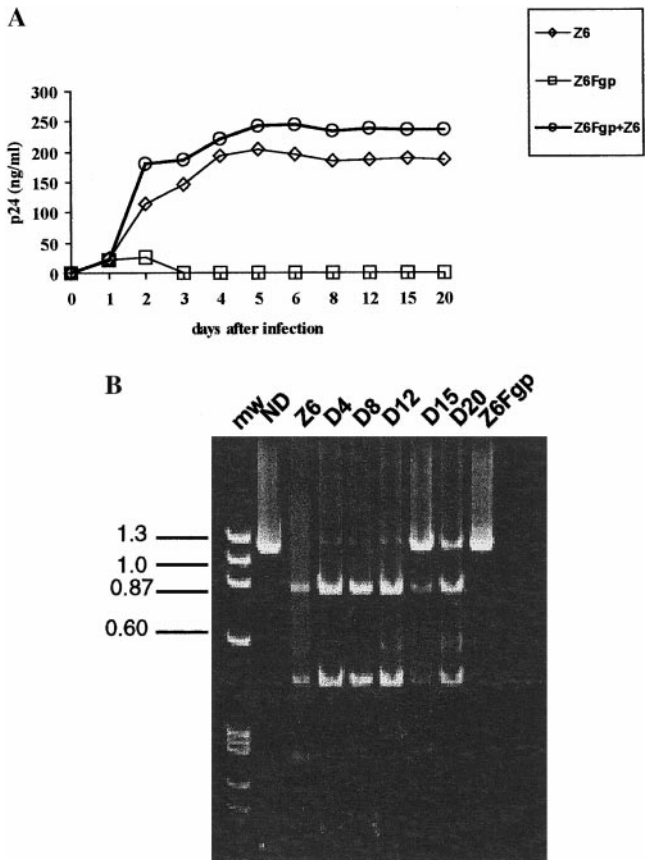

FIG. 6. Coinfection of PBMCs with recombinant (Z6Fgp) and parental (Z6) viruses. PBMCs were infected with a mixture of Z6Fgp and Z6 viral stocks at a rate of 10:1, respectively. (A) Several days after infection, the amount of virus release at cell supernatants was determined by an ELISA-p24 assay and compared with that of singly infected cultures. (B) The presence of each virus at the supernatant of double-infected culture was distinguished after *Eco*RI digestion of previously RT-PCR-amplified gp120 DNA fragments, because this sequence derived from the Z6 virus possesses an *Eco*RI restriction site that is absent in the sequence derived from the Z6Fgp virus. At 4, 8, 12, 15, and 20 days (D4, D8, D12, D15, and D20), an aliquot of the culture supernatant was collected and processed for RT-PCR amplification. MW indicates molecular weight marker ϕX174/*Hae*III (GIBCO BRL).

Z6Fgp-infected cells probably became superinfected with Z6 viruses, we were able to distinguish both viruses at the supernatant (Fig. 6B, lanes 6 and 7).

The phenotype of recombinant viruses could be reverted during the time of PBMC infection

Cultures of PBMCs infected with recombinant and parental viruses were maintained up to 60 days after infection, and at each 4-day interval, viral particle amounts at the supernatant were evaluated. We observed that viral production in recombinant virus-infected cells were very low after the first week of infection. However, in the supernatant of Z6Bgp-infected PBMCs, a burst in viral production was observed after 62 days postinfection. From this moment, the infectious process became highly productive, which suggested a spontaneous reversion of the Z6Bgp initial phenotype. In consequence, we searched for some mutations taking place at the *env* region that could account for this change in phenotype. We obtained the entire sequence of the *env* (gp120 and gp41 including *nef* gene) and p17 region from viruses present at each 4-day interval of PBMC culture through direct sequencing of PCR products. We identified, through sequence analyses of the revertant virus compared with that of the Z6Bgp, two amino acid substitutions in highly conserved regions of the gp120: a methionine-to-isoleucine substitution (M104I) in the C1 region and a glutamic acid-to-lysine substitution (E434K) in the C4 region (Fig. 7). We also looked at the amino acid sequences from the previous time points, and those sequences were equivalent to that of the Z6Bgp virus present at the beginning of infection. Finally, sequencing of the gp41 genes at all time points did not provide evidence of any changes in that gene (data not shown).

DISCUSSION

Given the high frequency of intersubtype recombinant HIV-1 strains being documented (Korber *et al.,* 1997), the main goal of this work was to access the *in vitro* biological behavior of mosaic viruses to look for ways of improving fitness. Because the envelope region of HIV-1 plays important roles in biological and immunological aspects of virus replication (Cann *et al.,* 1992; Cao *et al.,* 1993, 1996; Carrilo and Ratner, 1996; Cheng-Mayer *et al.,* 1988; Stamatatos and Cheng-Mayer, 1993), we attempted to construct the recombinant viruses within such region. The pZ6 infectious clone was successfully manipulated, and we obtained two types of recombinant constructs. The first set of recombinant viruses (Z6Bgp and Z6Fgp) possesses the entire gp120 region from B or F viral isolates, respectively, replacing the correspondent gene into the Z6 backbone. It has been reported that Vpu plays two crucial functions in HIV-1 life cycle: enhancement of virus release from infected cells and degradation of CD4/gp160 complexes at the endoplasmaic reticulum (RE) (Bour *et al.,* 1995, 1996; Bour and Strebel, 1996; Cucchirini *et al.,* 1995; Ewart *et al.,* 1996; Fujita *et al.,* 1996; Iwanati *et al.,* 1997; Klimkait *et al.,* 1990). Due to this, the second group of viruses [Z6Bgp(vpuZ6) and Z6Fgp(vpuZ6)] were generated by replacing the Z6 gp120-env with the correspondent region from the B or F isolates but with an intact ORF of Vpu protein, which overlaps at the beginning of gp120. HeLa cells transfected with all mosaic viruses released great amounts of virions, and the number of infectious particles measured by MAGI assay was the same as that of Z6 (Fig. 2 and data not shown). These results indicated that substitution of gp120 region in the Z6 infectious clone does not impose constrains to the formation of HIV-1 infectious particles in CD4-negative cell systems. Moreover, pro-

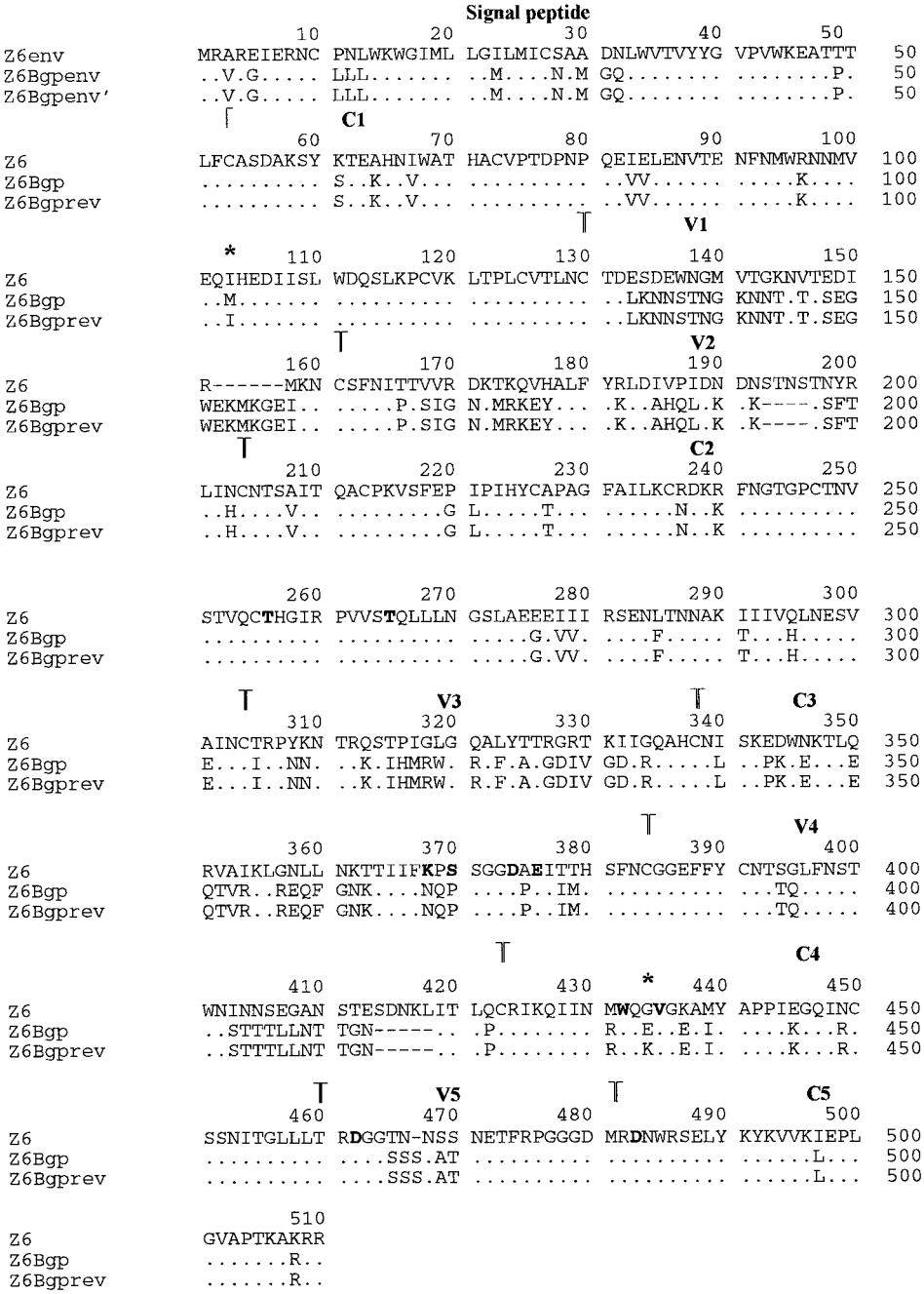

FIG. 7. Amino acid alignment of gp120 region of Z6, Z6Bgp, and the Z6Bgp revertant viruses. Sequence names are shown on left (Z6Bgprev indicates the Z6Bgp revertant virus). Sequences are numbered according to the Z6 sequence, and only changes relative to Z6 are shown in the alignment (dots represent similarity). Positions where mutations were found in the revertant virus are marked by asterisks. gp120 variable (V) and conserved (C) regions are indicated at the top. Amino acid residues critical for gp120 binding to CD4 are in bold.

tein pattern analysis of viral particles through immunoprecipitation with anti-HIV sera revealed the presence of both envelope glycoproteins gp120 and gp41 (Fig. 2B). Processing, glycosylation, and transporting of viral glycoprotein precursors throughout the cellular secretion pathway are indicative of the correct folding of such glycoproteins (Doms *et al.,* 1993; Willey and Strebel, 1996; Willey *et al.,* 1988, 1991). The HIV-1 gp160 precursor folding is a function of interactions between the gp120- and gp41-related regions (Earl *et al.,* 1990; Gelderblom *et al.,* 1987; Hunter, 1997; Leonard *et al.,* 1990; Otteken *et al.,*1996). The visualization of a normal protein pattern from the viral particles and the release of infectious particles from transfected cells confirm the efficiency of such processes even with the recombinant envelope.

Immunoprecipitation of gp41 using anti-gp120 antibodies was not observed for the recombinant viruses as it was for the Z6 (Fig. 3, compare lane 6 or 7 with lane 8).

This could be indicative of a weaker interaction between B- and F-related gp120 with the Z6 gp41 protein. It has been demonstrated that there is a decrease in viral infectivity for some HIV-1 isolates due to a loss of the gp120 surface glycoprotein (Gelderblom *et al.,* 1995; Mckcating *et al.,* 1991; Siciliano *et al.,* 1996). The weaker interaction described could represent an increase in shedding of the gp120 from the viral surface. However, it does not seems to have consequences for the viral particle formation in CD4-negative cells. The conformational status of the interacting gp120/gp41 in viral envelope affects the virus binding to the host cell receptor and the postbinding processes such as membrane fusion and virus entry (Bour *et al.,* 1995; Carrilo and Ratner, 1996; Helseth *et al.,* 1991; James *et al.,*1996; Moore *et al.,*1994; Stamatatos and Cheng-Mayer, 1993; Wang *et al.,* 1995; Wyatt and Sodroski, 1998). However, such recombinant viruses were perfectly able to enter CD4-positive cell lines (Fig. 4), indicating that a structural conformation of gp120 and gp41 proteins that allows the CD4 binding and viral entry was achieved.

Our results demonstrated that recombinant viruses behaved differently from parental viruses regarding the establishment of infections in CD4-positive cells. We observed a peak of virus production early in infection of recombinant viruses that dropped and established at very low levels (Fig. 3). The fact that recombinant viruses kill the same amount of cells at the early stage of infection as well as the parental viruses negates the idea that viral production could not be sustained due to massive cell death. Furthermore, viral DNA could be measured in recombinant virus-infected cells during all infectious periods, indicating that cells present in culture had been infected. The lack of virus release was not related to an inhibition of viral protein synthesis for at least 7 days after infection because the same levels of p24 expression in parental and recombinant Z6 virus-infected cells were detected. This is corroborated by the observation of a noteworthy cytopathic effect with formation of large syncytia at the seventh day of infection. Taking all of these characteristics together, we suggest that recombinant viruses are not able to sustain a productive infection in CD4-positive cells. The absence of virus release despite of viral structural protein expression could be related to a nonfunctional Vpu protein (Bour *et al.,* 1995, 1996; Bour and Strebel, 1996; Cucchirini *et al.,* 1995; Ewart *et al.,* 1996; Fujita *et al.,* 1996; Iwanati *et al.,* 1997; Klimkait *et al.,* 1990). The fact that Z6Bgp(vpuZ6) and Z6Fgp(vpuZ6) viruses have similar infectious characteristics of Z6Bgp and Z6Fgp viruses, which preserve the wild Vpu reading frame, rules out this possibility.

It is well established that the HIV-1 glycoprotein precursor gp160 has as important function during infection: the down-regulation of the viral receptor molecule CD4 (Bour *et al.,* 1991, 1995; Martin and Nayak, 1996c; Stevenson *et al.,* 1988). The major route responsible for the inhibition of expression of this molecule at the surface of infected HIV-1 cells is the binding of gp160 with CD4 during their syntheses in the endoplasmic reticulum (Crise and Rose, 1992; Crise *et al.,* 1990). The conserved amino acid residues critical for the extracellular gp120/CD4 attachment are also essential to gp160/CD4 interaction in the endoplasmic reticulum, but the achievement of a proper structural conformation seems to be critical for the intracellular binding of gp160 to CD4. It has been demonstrated that this proper conformation is a function of a correct interaction between gp120 and gp41 subunits within the gp160 precursor (Martin and Nayak, 1996a,b). The results presented here demonstrate that cells infected with recombinant viruses were not able to down-regulate CD4 expression in the cell surface compared with those infected with Z6 (Fig. 5). Thus the inefficiency of CD4 down-modulation in recombinant virus-infected cultures at 1 week postinfection probably could be accounted for by a blockage in the normal CD4/gp160 interaction.

Some hypotheses that attempt to correlate the blockage of CD4 down-regulation and the failure in establishment of productive infections were previously proposed. Marshal *et al.* (1992) demonstrated that a CD4 cell clone expressing 10% more CD4 molecules than the original cell line at the cell surface blocked HIV-1 viral replication at a postintegration stage. [Other enveloped viruses, such as influenza and coranaviruses, similarly developed a strategy of receptor down-modulation for efficient viral release (Palese *et al.,* 1974; Vlasak *et al.,* 1988).] Moreover, there are additional data demonstrating a straight correlation between high levels of CD4 expression at the infected cell surface and a failure to establish productive infection (Butera *et al.,* 1994). The simian immunodeficiency virus isolate SIV_{agm} is able to produce a stable infection in a low CD4-expressing cell line (Hut-78) but not in a high CD4-expressing cell line such as Sup-T1 (Hoxie *et al.,* 1988). Thus the differences in viral production between our chimerical viruses and the parental Z6 observed here could be accounted for by the fact that chimerical viruses are not able to down-regulate the CD4 receptor, consequently blocking virus release. In fact, this idea is supported by our coinfection experiment. In this case, the production of the Z6Fgp infection was allowed by coinfection of Z6Fgp nonproductively infected cells with Z6 parental virus. The *trans*-complementation experiment suggested that the lost intracellular function of our recombinant viruses could result from a defective gp160 chimerical protein that is complemented by the wild-type form, leading to the increase in viral production.

The spontaneous reversion of Z6Bgp phenotype after 62 days of infection highlights some aspects of recombinant virus infections. First, the occurrence of amino acid changes in the new virus demonstrate that although there is a lack of virus production, cells present in culture

were infected and had continuously undergone some cycles of virus replication and that mutations in conserved regions of gp120 (C1 and C4) could be indicative of selective pressure acting in this molecule. Second, the fact that amino acid changes involved a nonconservative mutation (lysine for glutamic acid, an amino acid residue that occurs in high frequencies at this site) within a region critical for gp120 binding to CD4 molecule strongly suggests that this mutation is a compensatory mutation for Z6Bgp recombinant. A nonconservative mutation (E434K) occurring at the C4 region very close to two amino acid residues (W/D432 and V435) that are fundamental to CD4 binding can account for the new phenotype, probably by promoting the correct function of gp160 precursor inside the endoplasmic reticulum. In fact, it has already been demonstrated that amino acid residues critical for the binding of gp120 to CD4 at the cell surface are also required for intracellular binding of gp160 to CD4; moreover, it appears that gp41 sequence may influence the way in which gp120 binds to CD4 (Martin and Nayak, 1996a,b). However, no mutations were found in gp41 or in other genomic regions of Z6Bgp, like *nef* and p17. Thus the impairment of chimerical gp160 to bind CD4 intracellularly can be a consequence of inadequate gp120/gp41 conformation, masking the correct amino acid residues important for this attachment. Wang *et al.* (1996) demonstrated the importance of single mutations in C1 and C4 regions that account for phenotype reversion of HIV-1 isolates impaired with CD4 binding, suggesting a change in functional interactions as the consequence of these compensatory mutations.

Structural impairments between the gp120 and gp41 molecules can thus have a profound impact on gp120 functions (deMareuil *et al.,* 1995, 1992; Helsethe *et al.,* 1991, 1992). We suggest here that gp160-CD4 downmodulation is an important event in the establishment of productive infections in CD4-positive infected cells. Nevertheless, this selective pressure could exist to preserve the correct gp120/gp41 interactions. Thus recombination in HIV genomes within this region, bringing together interacting molecules with diverse genetic identities, will be successful only if these molecules can interact properly.

MATERIALS AND METHODS

Cells

HeLa cell lines were maintained as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, penicillin/streptomycin [(P/S) 500 U/500 mg/ml], and 2 mM L-glutamine at 37°C with 5% CO_2. Another HeLa cell line, termed HeLa MAGI, was maintained as described but the medium was complemented with hygromicin B (0.1 mg/ml) and G418 (0.15 mg/ml) antibiotics (Kimpton and Emerman, 1992).

CEM, Hut-78, and MT-2 cells were maintained as suspension cultures in RPMI 1640 medium supplemented with 10% FBS, P/S (5000 U/10 mg%), and 2 mM L-glutamine. PBMCs were isolated from the whole blood of healthy donors using the Phycol-Hypaque density separation medium (Organon Teknika, Netherlands) and centrifugation. The PBMCs were then stimulated with 1 μg/ml PHA and cultured in RPMI 1640 medium supplemented with 10% FBS, P/S (500 U/500 mg/ml), and 2 mM L-glutamine for 3 days, before culture infections. At the moment of use, the medium was replaced by a medium containing 20 U/ml recombinant IL-2.

Virus

Brazilian viral isolates used in this study had been previously characterized (Ramos *et al.,* 1999.). BR48 isolate was subtyped as B in both *gag* and *env* genes, whereas BR59 isolate were subtyped as F in both genomic regions. Also, those isolates were both characterized for coreceptor use as R5 and X4 dual tropic viruses and showed a syncytia-inducing (SI) phenotype in CEMss cell cultures (data not shown). Culture supernatants were analyzed for virus production by ELISA-p24 specific assays (Coulter, Krefeld, Germany), and the infected cell-free supernatants were stored at −70°C. To obtain DNA samples for further PCR amplification reactions, infected cells were treated with the Tris–Triton–PTK lysis buffer and incubated at 65°C for 1 h after PTK inactivation for 10 min at 95°C (Pianiazek *et al.,* 1991). Lysates were stored at −70°C.

Plasmids, cloning strategies, and sequencing

pZ6, a pBR322 containing the full-length genome of a clade D HIV-1 isolate (Srinivasan *et al.,* 1987), was used to generate the chimeras. PCR-amplified gp120 *env* fragments from Br48 or Br59 isolates were cloned into pCRII (TA Cloning Kit Vector; InVitrogen, San Diego, CA). The clones were further used as donors of the regions to be replaced in the infectious clone pZ6. The strategy to obtain the chimeric infectious clones used PCR amplification of both vector backbone without the region to be replaced and the *env* region from the donor DNAs. Primers that were used contain a type II endonuclease site, *Eam*1104I, to generate, after enzymatic digestion of the amplified product, a complementary tail at the 5′ and 3′ ends of insert and vector fragments, which on further treatment with T4 ligase can reconstitute an entire molecule (Padgett and Sorge, 1996). The PCR conditions for insert and vector amplifications basically followed the standard protocol (Seamless Cloning Kit; Stratagene, La Jolla, CA). All PCRs were performed in presence of modified dCTP-methyl dideoxynucleotide to protect from digestion the internal *Eam*1104I sites that could exit those molecules. The primers used to substitute the entire gp120 gene are depicted in Table 1. PCR products were then purified using Qiagen QUIAquick Spin Kit (Qiagen,

TABLE 1

Primers Used in the pZ6 Manipulation

Primer	Position in Z6 clone	Sequence (5′ to 3′)[a]	Region replaced
gp120F	6227–6251	AGTTA CTCTTCA **TGA** GAGTGAGGGGGATAGAGAGGA	gp120 amplification of donor sequence
gp120R	7753–7732	AGTTA CTCTTC T **GCT** CTTTTTTCTCTTTCCACCACT	
Vector 120F	7749–7773	AGTTA CTCTTC G **AGC** AATAGGACTAGGAGCTATTGTT	Vector amplification but gp120 region
Vector 120R	6231–6207	AGTTA CTCTTC C **TCA** TTGCCACTGTCTTCTGCTCTT	
gp120(−vpu)F[b]	6562–6587	AGTTA CTCTTC C **AAA** GCCTAAAACCATGTGTTAAAAT	gp120 amplification of the donor but Vpu
Vector 120(+vpu)R[b]	6567–6543	AGTTA CTCTTC C **TTT** GATCCCATAAACTGATTATAT	Vector amplification but gp120 region (keeping Z6 Vpu)

[a] The underlined nucleotides represent the recognition site for *Eam*1104I endonuclease, and the bold nucleotides represent the complementary ends generated after enzymatic digestion of PCR amplified products. The insert forward primer end is reverse complementary to the vector reverse primer end, in the same way as the insert reverse primer end and the vector forward primer end.

[b] To substitute the gp120 gene in the pZ6 without changing the second half of Vpu coding sequence that overlaps, another forward primer in combination with the reverse already described and another reverse primer in combination with the forward previously described were used for the insert and the vector, respectively.

Hilden, Germany) according to the manufacturer's specifications. Related purified products were combined in the same reaction tube, with a fivefold excess of insert compared with the vector, in the presence of T4 DNA ligase and endonuclease *Eam*1104I according to the protocol. The reaction was incubated at 37°C for 1 h. One tenth of the ligation reaction was then used to transform the XL1-Blue MRF′ supercompetent bacteria supplied with the kit. A schematic representation of all constructs is shown in Fig. 1.

Sequencing of plasmids or PCR products was performed with a dye-labeled dideoxynucleotide mix (ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit; Applied Biosystems, Foster City, CA) and run in an ABI 373 automated sequencer.

Mammalian cell transfections

To obtain infectious viral particles from the chimeric clones, HeLa cells were transfected with the LipofectAMINE cationic liposome-complexed plasmids (GIBCO BRL, Gaithersburg, MD). Briefly, 2×10^5 cells were seeded onto each well of 6-well plate 18 h before transfection and maintained as previously described. One microgram of each plasmid was combined with 10 μl of the liposome solution in 500 μl of medium 30 min before addition to the cells. Cells were incubated with the DNA–liposome complexes at 37°C for 5 h, fresh medium was added to a total volume of 2 ml, and cells were incubated at 37°C overnight. On the next day, the medium was replaced by fresh medium containing FBS, and aliquots from the supernatants were harvested for each 24-h period and analyzed for the presence of viral particles using the ELISA-p24 assay (Coulter, Krefeld, Germany). Supernatants harvested from transfected cells at the peak of viral production were clarified from cell debris by centrifugation at 10,000 $\times$ *g* for 1 h, and 1-ml aliquots were stored at −70°C. Each frozen aliquot was thawed only once before use.

Protein labeling and radioimmunoprecipitation

HeLa-transfected cells were starved for 1 h in methionine–cysteine-free medium before being labeled for 12 h in medium containing 350 μCi of ^{35}S-methionine-cysteine (*Trans*Label System; ICN, Costa Mesa, CA). At the end of the radioactive labeling period, the supernatants were collected and layered on a 60% sucrose cushion, and the viral particles were pelleted at 35,000 rpm for 2 h. Radioimmunoprecipitation assays (RIPAs) were performed as described elsewhere. Briefly, the pellets were resuspended in 1.5 ml of RIPA buffer (150 mM NaCl, 100 mM Tris–HCl, pH 7.5, 5 mM EDTA, 1% NaDOC, 1% Triton X-100, 1% SDS, 10 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml benzamidin, 10 μg/ml TLCK, and 0.2 mM PMSF) and incubated on ice for 15 min. Five hundred microliters were incubated with 5 μl of an anti-HIV serum or with 5 μl of anti-gp120 or anti-gp41 antibodies overnight at 4°C. Then, 5 μl of a *Staphylococcus aureus* protein A-Sepharose suspension was added, and the mixture was incubated for 2 h at 4°C. The immunocomplexes were washed three times with RIPA buffer, suspended in reducing sample buffer, and boiled for 5 min before loaded in a 4% stacking/12.5% separating SDS–

polyacrylamide gel. Gels were dried and subjected to autoradiography.

Virus titration

Virus titration assays were preformed in MAGI cells as previously described (Kimpton and Emerman, 1992). Briefly, supernatants of transfected cells, previously monitored for the presence of viral particles by ELISA-p24 assay (Coulter), were used for serial dilutions in DMEM, and those dilutions were layered on CD4-LTR/β-gal HeLa (MAGI) cell monolayers (at 20% confluence) in the presence of 20 μg/ml DEAE-dextran. Cells were cultivated in DMEM with 10% FBS for 2 days. Cells were then fixed and stained with X-Gal according to the standard protocol (Kimpton and Emerman, 1992). Blue cells were counted under a microscope at a magnification of 100×.

PBMCs and established lymphocyte infections

After 3 days in the presence of PHA stimulation, 1×10^7 PBMCs were pelleted and resuspended in a 2-ml volume of RPMI 1640 with 1×10^5 $TCID_{50}$ viral particles (m.o.i. = 0.1). After 3 h of viral absorption at 37°C, the medium was removed, the cells were washed twice with PBS suspended in a 1–0ml volume of RPMI 1640 containing 10% FBS and 20 U/ml IL-2, and incubated at 37°C. For every 4-day period, half of the cell culture volume was taken and processed for supernatant and cell lysate evaluation and storage. The same volumes of healthy PHA-stimulated PBMCs in a medium with 20 U/ml IL-2 were added to the cultures.

CEMss and Hut-78 cells were infected at 37°C at an m.o.i. of 0.1, with a 3-h absorption period. Next, the medium was removed, and the cells were washed twice with PBS and suspended in RPMI 1640 medium with 10% FBS. Infected cells were evaluated daily under a microscope to observe the cytophathic effects.

Virus entry assays

To evaluate the virus efficiency for CD4 absorption and entry, a suspension of CEMss cells was incubated for 3 h with a minimum volume of RPMI 1640 medium containing viral particles at an m.o.i. of 1 at both 4°C and 37°C. The former temperature allows virus binding to the cell surface CD4, whereas the letter temperature allows both binding to CD4 and virus entry into the cell cytoplasm. After the absorption period, the cells were washed with PBS, and the cells infected at 4°C were directly suspended in the ELISA lysis buffer (Aiken and Trono, 1995). As a control, samples were also assayed at 4°C, followed by trypsin treatment before lysis and ELISA-p24 detection. Cells infected at 37°C were suspended in a PBS–0.5 M EDTA solution containing 0.25% of trypsin and incubated for 30 min at 37°C to eliminate virus particles that were bound to the cell surface. Then cells were pelleted and directly suspended in the ELISA lysis buffer. All lysates were used for viral particle detection by ELISA-p24-specific assays (Coulter).

PCRs

Lysates from infected cultures were used in nested PCRs for *env* amplification. Reactions were performed as described elsewhere. Primers used for *env* amplification were outer ED3/ED14 and inner ED5/ED12 (Delwart *et al.,* 1994). Quantitative PCRs were performed using DNA limiting dilution protocol as previously described (Simmonds *et al.,* 1990). The supernatant of infected cells was used for viral RNA extraction with the QIAamp RNA Extraction Kit (Qiagen). RNAs were then used as templates for MuLV RT-PCRs. The procedure was performed as described in the GeneAmp RNA PCR Kit Protocol (Perkin–Elmer Cetus, Norwalk, CT).

Cell surface CD4 expression analysis

Expression of CD4 in the surface of CEMss cells was evaluated by FACS analysis. Briefly, 2×10^6 infected or mock-infected cells were incubated in the dark with FITC-conjugated anti-CD4 monoclonal antibodies for 30 min at room temperature. After that, cells were washed with PBS containing 2% human serum, 00.2% sodium azide, and 0.1% BSA. Cells were fixed overnight in 1% formaldehyde before FACS analysis in a Becton Dickinson FACSscan with CELLQuest software (San Jose, CA).

Samples were taken of cells from infected cultures and used to confirm the presence of viral DNA by quantitative PCR. Results demonstrated for all infected cultures (Z6, Z6Bgp, and Z6Fgp) that each cell in the infected population harbored viral DNA, confirming that cells analyzed by FACS had been previously infected.

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