JOURNAL OF VIROLOGY, May 1993, p. 2807–2814
0022-538X/93/052807-08$02.00/0

Vol. 67, No. 5

Restricted Replication of Simian Immunodeficiency Virus Strain 239 in Macrophages Is Determined by *env* but Is Not due to Restricted Entry

KAZUYASU MORI,[1] DOUGLAS J. RINGLER,[2] AND RONALD C. DESROSIERS[1]*

Division of Microbiology[1] and Division of Pathology,[2] New England Regional Primate Research Center, Harvard Medical School, One Pine Hill Drive, Box 9102, Southborough, Massachusetts 01772-9102

Received 14 December 1992/Accepted 19 January 1993

Virus derived from the infectious, pathogenic, molecular clone of simian immunodeficiency virus (SIV) called SIVmac239 replicates poorly in primary rhesus monkey alveolar macrophage cultures. Variants with three to nine amino acid changes in the envelope replicate 100 to 1,000 times more efficiently in these macrophage cultures than parental SIVmac239. Early events, including virus entry into cells, were analyzed by measuring the amounts of newly synthesized viral DNA 14 to 16 h after infection of macrophages by using a quantitative polymerase chain reaction method. SIVmac239 was found to enter macrophages with an efficiency similar to that of the macrophage-tropic derivatives. The assay indeed measured newly synthesized viral DNA since detection was inhibited by the reverse transcriptase inhibitors azidothymidine and foscarnet and by heat inactivation of the virus stock prior to infection. Furthermore, entry of SIVmac239 and macrophage-tropic variant into macrophages was inhibited by monoclonal antibody against CD4. Analysis of the time course of viral DNA accumulation showed that although initial entry of SIVmac239 into cells occurred normally, subsequent logarithmic increases in the amounts of viral DNA associated with spread of virus through the macrophage cultures was blocked. Increasing the amount of SIVmac239 incubated with macrophages increased the amount of virus entering the cell, but this could not overcome the block to replication. Thus, restricted replication of SIVmac239 in macrophages is determined by the envelope, but surprisingly it is not due to restricted virus entry.

CD4$^+$ T lymphocytes are major targets of human immunodeficiency virus type 1 (HIV-1) infection in vivo, and depletion of these cells is clearly a major factor in the development of AIDS in humans. However, cells of mononuclear phagocytic lineage, including monocytes in blood, microglial cells in the brain, and macrophages in other tissues, are also targets of HIV-1 infection in vivo (1, 16, 21, 22, 25, 51). The more primitive ungulate lentiviruses do not target CD4$^+$ lymphocytes but replicate and persist primarily in macrophages (for reviews, see references 5, 34, and 50). It is currently not clear whether the infection of monocytes/macrophages by HIV-1 is simply an evolutionary remnant or is an essential element for HIV-1 persistence, dissemination, or disease progression.

Since simian immunodeficiency virus (SIV) infection of rhesus monkeys mimics many of the important features of HIV-1 infection of humans, it is an important model for the study of AIDS pathogenesis. SIVmac, like HIV, uses the CD4 protein as its initial receptor (7, 18, 28), has a similar genome organization (4, 10, 12, 13), infects both lymphocytes and macrophages (2, 8, 14, 15, 31, 39, 47), and is able to cause AIDS in the common rhesus monkey (10, 12, 13, 19, 20, 24). Virus derived from the infectious, pathogenic, molecular clone SIVmac239 replicates poorly in macrophages (11, 31). Variants competent for replication in macrophages evolved during the course of infection of several rhesus monkeys with cloned SIVmac239, and the emergence of these macrophage-competent variants was associated with the development of a characteristic encephalitis and giant-cell pneumonia (11, 19, 31). Sequence changes in the envelope gene (*env*) were found to be the primary determinants of the replicative capacity of these variants for macrophages (31). Ability to replicate well in macrophages can be imparted by as few as three or as many as nine amino acid changes in the envelope glycoprotein (31). None of the contributing changes were located in the region of SIVmac that corresponds to HIV-1 V3, a domain of the gp120 glycoprotein that can contribute to HIV-1 macrophage tropism (17, 35, 44, 48).

The block to replication of at least one HIV-1 strain (NL4-3) in human macrophages has been previously localized to early events that include virus entry into the cell (35). However, one recent study concluded that two strains of HIV-1 that replicate very poorly in macrophages are still able to enter these cells and retrotranscribe their genomes (43). We analyzed the ability of several cloned SIVs to enter rhesus monkey macrophages. SIVmac239 was found to enter macrophages with an efficiency similar to that of the closely matched variants with high replicative capacity for macrophages. Thus, although sequences in *env* are responsible for the deficient replication of SIVmac239 in macrophages, the block to SIVmac239 replication in macrophages is located at some step subsequent to virus entry.

MATERIALS AND METHODS

Plasmids. SIVmac239 proviral DNA has been subcloned as two segments, p239SpSp5′ and p239SpE3′ (19). *env* gene sequences of p239SpE3′ have been replaced with corresponding sequences of SIVmac316 to construct macrophage-competent recombinant viruses. SIVmac239/316ENV, SIVmac239/316SU, and SIVmac239/316TM contain the entire

* Corresponding author.

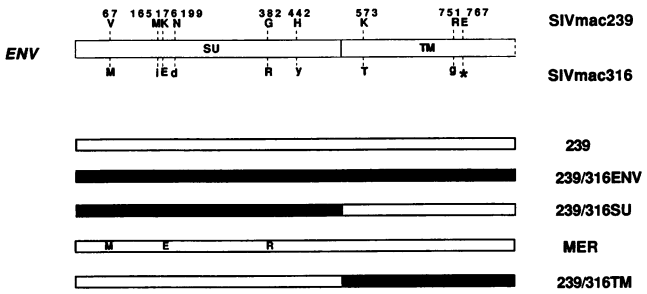

FIG. 1. Envelope sequences of cloned viruses. Numbers indicate the amino acid number in *env* (31). Amino acid changes at positions 67, 176, 382, 573, and 767 have been shown to contribute to the macrophage tropism of SIVmac316.

envelope, SU portion, and TM portion, respectively, of the macrophage-competent SIVmac316 (31). A plasmid which encodes a virus called MER having only three amino acid changes (V-67→M, K-176→E, and G-382→R) in *env* was constructed by using restriction enzyme sites *Hin*dIII (nucleotide [nt] 6239), *Dra*I (nt 7505), and *Ban*I (nt 8158) (31). Nucleotide and *env* amino acid numbering systems are as previously described (31, 38).

Virus. SIVmac239 and its derivatives were prepared by transfection of CEMx174 or COS1 cells with DEAE-dextran as previously described (9, 32). Virus stocks containing high infectious titers were prepared from culture supernatants of CEMx174 cells 7 to 10 days after transfection or by infection with virus stocks prepared by transient expression in transfected COS1 cells. Culture supernatants obtained near the peak of virus production were harvested, and aliquots of filtered (pore size, 0.45 μm) supernatant were stored at −70°C. Virus stocks used for evaluation of virus entry were subjected to DNase I digestion (5 μg/ml; Worthington, Freehold, N.J.) for 30 min at room temperature in the presence of 0.01 M $MgCl_2$ before filtration. COS1 virus stocks were prepared from culture supernatants of the cells 2 to 3 days posttransfection. The amount of virus was

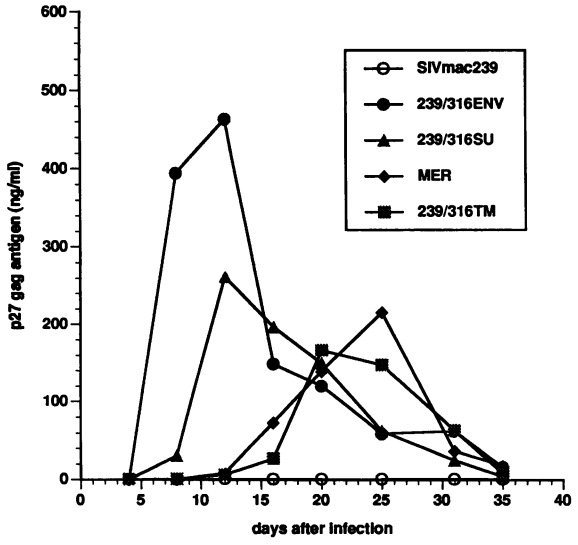

FIG. 2. Virus production in alveolar macrophages. Virus stocks containing 0.1 ng of p27 *gag* antigen harvested 3 days after transfection of COS1 cells were used to infect 3 × 10^5 cells.

standardized by assay of $p27^{gag}$ antigen with a commercial antigen capture kit (Coulter Corp., Hialeah, Fla.) or by determination of 50% tissue culture infectious doses ($TCID_{50}$) in CEMx174 cells.

Cells. The human T-B hybrid cell line CEMx174 was maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Alveolar macrophages were obtained from bronchoalveolar lavage specimens from healthy, mature rhesus macaques that were serologically negative for SIV, type D retrovirus, and simian foamy virus. At least 95% of the cells were demonstrated to be macrophages on the basis of morphologic criteria, adherence to plastic flasks, cell surface markers, and opsonizing activity, as described previously (11, 39, 46). Macrophages were maintained in Iscove's modified Dulbecco's medium supplemented with 5% heat-inactivated fetal calf serum, 10% human type AB serum (GIBCO, Grand Island, N.Y.), 100 U of penicillin per ml, 100 μg of streptomycin per ml, 10 μg of gentamicin per ml, and 0.25 μg of amphotericin B per ml at a concentration of 5 × 10^5 cells per ml in 24- or 48-well flat-bottom plates. The culture medium was changed twice weekly.

Quantitative PCR method for detection of newly synthesized SIV DNA. The procedure used for quantitating newly synthesized viral DNA was adapted from that originally described by Zack et al. (49) and by Cann et al. (3). Macrophages or CEMx174 cells (2 × 10^5 to 10 × 10^5) were infected with DNase-treated SIV at 37°C for 2 h. Cells were washed to remove residual virus and incubated for the indicated times. At the time of harvest, macrophages were detached with 0.5 mM EDTA in phosphate-buffered saline (PBS), washed twice with PBS, lysed in urea lysis buffer (4.7 M urea, 1.3% [wt/vol] sodium dodecyl sulfate, 0.23 M NaCl, 0.67 mM EDTA [pH 8.0], 6.7 mM Tris-HCl [pH 8.0]), and extracted with phenol-chloroform, and the DNA was precipitated with ethanol (49). To avoid extraneous contamination with SIV DNA, DNA isolation and polymerase chain reactions (PCRs) were performed as suggested for highly sensitive PCR methods (23). To detect SIV DNA, we used two sets of primers: for R/U5 sequences, M95 (nt 535 to 555) (5′-GGCTGGCAGATTGAGCCCTGG-3′) and M96 (nt 714 to 694) (5′-GATGGGAACACACACTAGCTT-3′); for *gag* sequences, M97 (nt 1065 to 1085) (5′-AACTCCGTCTTGTC AGGGAAG-3′) and M98 (nt 1206 to 1186) (5′-CCAACAGG CTTTCTGCTAATC-3′). For normalization, β-globin DNA was amplified with primers 5′-ACACAACTGTGTTCA CTAGC-3′ and 5′-CAACTTCATCCACGTTCACC-3′ (42, 49). One oligonucleotide of each primer pair was end labeled with ^{32}P by using T4 polynucleotide kinase. Each PCR contained 0.25 mM each deoxynucleoside triphosphate, 50 mM KCl, 10 mM Tris HCl (pH 8.3), 6 mM $MgCl_2$, 0.1 mg of

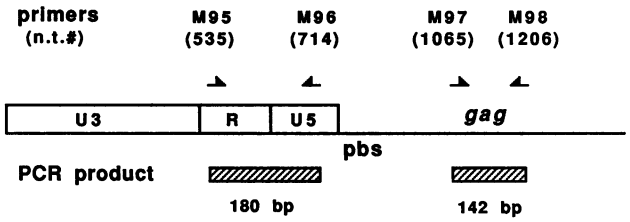

FIG. 3. Primers and PCR products used for analysis of newly synthesized SIV DNA. Nucleotide numbers (n.t.#) are from the published SIVmac239 sequence (38). Abbreviation: pbs, lysyl-tRNA primer-binding site.

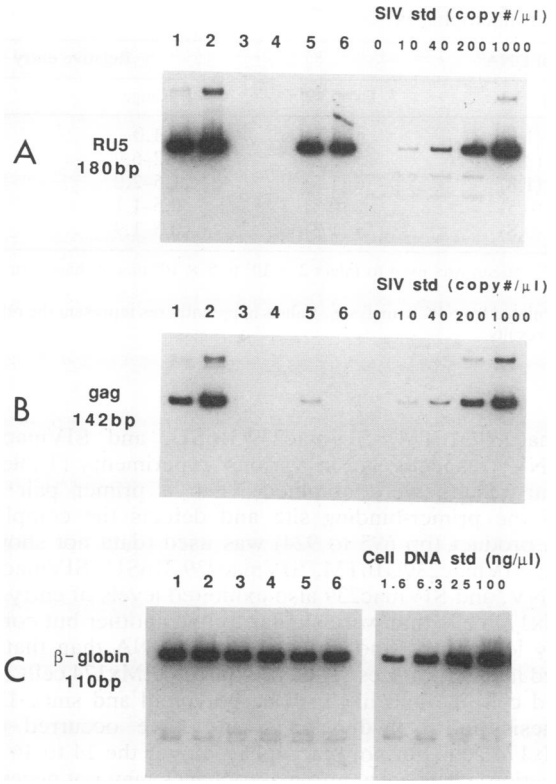

FIG. 4. PCR analysis of newly synthesized SIV DNA in macrophages. Total-cell DNA was prepared from alveolar macrophages (10^6 cells) infected with SIVmac239 or SIVmac239/316ENV 16 h after infection with 2×10^5 $TCID_{50}$ (230 and 480 ng of $p27^{gag}$ antigen, respectively). To confirm that the detected viral DNA was newly synthesized, we added 50 μM AZT or 300 μM PFA to some cultures starting 7 h before infection. SIV DNAs corresponding to RU5 (A), *gag* (B), and β-globin DNA (C) were separately amplified by PCR and analyzed by polyacrylamide gel electrophoresis. Lanes: 1, SIVmac239; 2, SIVmac239/316ENV; 3, heat-inactivated (60°C for 30 min) SIVmac239; 4, heat-inactivated SIVmac239/316ENV; 5, SIVmac239/316ENV with AZT; 6, SIVmac239/316ENV with PFA. SIV standards (0, 10, 40, 200, and 1,000 copies per μl) and cellular DNA standards (1.6, 6.3, 25, and 100 ng/μl) were used for quantitation.

bovine serum albumin per ml, 1×10^6 to 5×10^6 cpm of primer, 50 ng of unlabeled second primer, 1.25 U of *Taq* DNA polymerase (Perkin-Elmer, Norwalk, Conn.), and 2.5 μl of sample or standard DNA. The reaction mixture (25 μl) was overlaid with 50 μl of mineral oil and subjected to 30 cycles of amplification (denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 1 min) in a Perkin-Elmer thermocycler. PCR products were analyzed by polyacrylamide gel electrophoresis (7% polyacrylamide) and visualized by autoradiography of the dried gel. Amounts of DNA were quantitated by Cerenkov counting. SIV standards (10 to 10^4 DNA copies per μl) were prepared by dilution of the linearized p239SpSp5′ plasmid with 0.5 μg of rhesus monkey DNA.

The monoclonal antibody (MAb) 19thy5D7 (anti-T4A), which was previously shown to block SIVmac251 infection of rhesus monkey peripheral blood lymphocytes (18), was kindly provided by S. Schlossman, Dana-Farber Cancer Institute, Boston, Mass. Alveolar macrophages (2×10^5 cells), preincubated for 30 min at 37°C with medium containing 500 μg of human immunoglobulin G (IgG) (Sigma Chemical, St. Louis, Mo.) per ml, were incubated with 19thy5D7 or negative control pooled MAb for 30 min at 37°C and then infected with either SIVmac239 or SIVmac239/316ENV (50 and 70 ng of $p27^{gag}$, respectively) for 2 h at 37°C. The cells were washed with PBS and kept in culture for 13 h.

RESULTS

Replication of SIVmac239 and recombinant viruses. Of nine amino acid changes in the envelope glycoprotein of the SIVmac316 variant, at least five contribute to the ability to replicate well in macrophages (31). These five are V-67→M, K-176→E, G-382→R, K-573→T, and E-767→Stop (Fig. 1). Four recombinant viruses were used for the present study (Fig. 1). SIVmac239/316ENV contained all nine amino acid changes; SIVmac239/316SU contained the six amino acid changes in SU (gp120); SIVmac239/316TM contained the three amino acid changes in TM (gp41); MER contained only V-67→M, K-176→E, and G-382→R. SIVmac239 and the recombinant viruses were produced by transient expression in transfected COS cells and examined for their ability to replicate in rhesus monkey alveolar macrophages. All four recombinant viruses replicated at least 100-fold more efficiently than SIVmac239 in these macrophage cultures (Fig. 2). All viruses replicated well in CEMx174 cells and in rhesus monkey peripheral blood mononuclear cell cultures (data not shown). The results with SIVmac239, SIVmac239/316ENV, SIVmac239/316SU, and SIVmac239/316TM are quite similar to those that we previously reported (31).

Quantitation of virus entry. Since glycoproteins encoded by *env* are important for retroviral entry into target cells, we investigated the effects of these envelope changes on early events that include virus entry into cells. Upon entry into cells, retroviruses reverse transcribe their genomic RNA into DNA. We therefore quantitated the amounts of newly synthesized viral DNA 14 to 16 h after exposure of macrophages to the virus. SIV DNA was amplified from cellular DNA with R/U5 primers (180-bp product) or *gag* primers (142-bp product) (Fig. 3). β-Globin DNA was used to normalize DNA detection in the assay. To verify that this procedure indeed detects newly synthesized viral DNA, we added the reverse transcriptase inhibitor azidothymidine (AZT; 50 μM) or phosphonoformic acid (PFA; 300 μM) to some of the cultures 7 h prior to infection (29, 41). The replication of SIVmac239/316ENV in macrophages was sup-

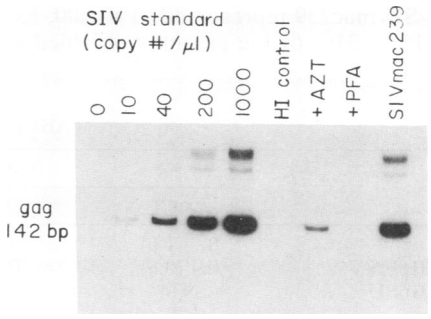

FIG. 5. Synthesis of SIVmac239 *gag* DNA is also sensitive to RT inhibitors. Macrophages (2×10^5) were incubated with PFA (300 μM) or AZT (50 μM) 7 h prior to infection with SIVmac239 containing 50 ng of p27. Total-cell DNA was prepared 15.5 h after infection and analyzed for the amount of SIV *gag* DNA. HI, heat inactivation of virus stocks.

TABLE 1. SIV entry into alveolar macrophages[a]

Virus	Amt of newly synthesized viral DNA[b]				Relative entry	
	Expt 1	Expt 2	Expt 3-1	Expt 3-2	Range	Avg
SIVmac239	9 (1.0)	31 (1.0)	20 (1.0)	4 (1.0)	1.0	1.0
SIVmac239/316ENV	62 (6.9)	100 (3.2)	67 (3.4)	15 (3.8)	3.2–6.9	4.3
SIVmac239/316SU	NT[c]	61 (2.0)	35 (1.8)	6 (1.5)	1.5–2.0	1.7
MER	NT	34 (1.1)	10 (0.5)	2 (0.5)	0.5–1.1	0.7
SIVmac239/316TM	3 (0.3)	59 (1.9)	15 (0.8)	5 (1.3)	0.3–1.9	1.1

[a] Virus containing 100 ng (experiment 1, 2, and 3-1) or 20 ng (experiment 3-2) of $p27^{gag}$ antigen was used to infect 2×10^5 to 5×10^5 macrophages for 15 h. Virus entry was compared by measuring the amounts of newly synthesized viral DNA.

[b] Amounts of viral DNA represent the copy number per nanogram of total-cell DNA calculated from PCR analyses. Values in parentheses represent the relative amount of newly synthesized viral DNA in macrophages compared with the SIVmac239 results.

[c] NT, not tested.

pressed to basal levels for 30 days by these reverse transcriptase inhibitors (data not shown). Both inhibitors blocked more than 95% of *gag* DNA detected by this assay (Fig. 4B, lanes 5 and 6 for SIVmac239/316ENV and Fig. 5 for SIVmac239). PFA produced more complete inhibition of *gag* DNA synthesis than AZT at the concentrations used. PFA and AZT did not, however, efficiently lower the amounts of R/U5 DNA detected by this assay (Fig. 4A, lanes 5 and 6). The poor inhibition of R/U5 DNA detection observed with these inhibitors is probably due to the close proximity of this region to the natural lysyl-tRNA primer-binding site used for initiation of DNA synthesis by reverse transcriptase in vivo (Fig. 3). Near-normal synthesis of the very short products containing R/U5 in the presence of AZT and PFA is not really surprising, since both AZT-triphosphate and PFA are competitive inhibitors. AZT-triphosphate is a competitive inhibitor of dTTP incorporation as well as a chain terminator, and PFA competes for the PP_i-binding site. Short reverse transcripts containing R/U5 may also be present or synthesized within the virion itself (27, 45). In addition to the dramatic inhibition of *gag* DNA synthesis in this assay caused by PFA and AZT, heat inactivation of the virus stock at 60°C for 30 min eliminated detection of viral DNA (Fig. 4A and B, lanes 4). The *gag* primers were thus used to quantitate the amounts of newly synthesized viral DNA using this assay.

Virus entry into macrophages was next assessed for SIVmac239 and the four variants by using this assay. The amount of virus used for incubation with macrophages was normalized either by the number of $TCID_{50}$ (Fig. 4, 6, and 7) or by the amount of $p27^{gag}$ antigen (Tables 1 and 2). SIVmac239 entry into macrophages was surprisingly efficient. The amounts of newly synthesized viral DNA obtained with SIVmac239 represented 91 to 200, 53 to 300, 50 to 67, and 15 to 31% of the amounts obtained with MER, SIVmac239/316TM, SIVmac239/316SU, and SIVmac239/316ENV, respectively, in various experiments (Table 1). Similar results were obtained when a primer pair that spans the primer-binding site and detects the completed DNA product (bp 695 to 924) was used (data not shown). MER, SIVmac239/316TM, SIVmac239/316SU, SIVmac239/316ENV, and SIVmac239 also exhibited levels of entry into CEMx174 cells that were similar to one another but considerably lower per nanogram of cellular DNA than that observed in macrophages (Table 2). Since CEMx174 cells are a hybrid cell line and likely to be polyploid and since DNA synthesis and cell division would have occurred with CEMx174 cells but not macrophages over the 14 to 16 h of incubation, virus entry into macrophages may not necessarily be more efficient on a per cell basis.

Restricted replication of SIVmac239 in macrophages follows entry. Viral DNA accumulation over longer periods was studied first under conditions of infection with equal $TCID_{50}$. With SIVmac239/316ENV, the level of viral DNA in macrophages increased dramatically, approximately 22-fold, between 14 and 66 h after infection (Fig. 6). In contrast, SIVmac239 showed no such dramatic increases in viral DNA accumulation over the initial levels.

Viral DNA accumulation was next studied under conditions where much more SIVmac239 (3×10^4 $TCID_{50}$) than SIVmac239/316ENV (3×10^3 $TCID_{50}$) was used. The amount of viral DNA in macrophages with SIVmac239 increased to high levels early but again did not increase at later times (Fig. 7A). At early time points, macrophages with SIVmac239/316ENV contained significantly less newly synthesized viral DNA than in the SIVmac239 cultures, consistent with the lower multiplicity of infection (Fig. 7A). However, the amounts of SIVmac239/316ENV viral DNA increased dramatically with time, and this increase was

TABLE 2. SIV entry into CEMx174 cells[a]

Virus	Amt of newly synthesized viral DNA[b]				Relative entry	
	Expt 1	Expt 2	Expt 3	Expt 4	Range	Avg
SIVmac239	0.7 (1.0)	0.3 (1.0)	0.5 (1.0)	1.4 (1.0)	1.0	1.0
SIVmac239/316ENV	0.4 (0.6)	0.2 (0.7)	1.1 (2.2)	0.4 (0.3)	0.3–2.2	1.0
SIVmac239/316SU	NT[c]	NT	0.3 (0.6)	0.7 (0.5)	0.5–0.6	0.6
MER	NT	0.3 (1.0)	0.3 (0.6)	0.9 (0.6)	0.6–1.0	0.7
SIVmac239/316TM	1.1 (1.6)	NT	1.8 (3.6)	2.3 (1.6)	1.6–3.6	2.3

[a] Virus containing 100 ng of $p27^{gag}$ antigen was used to infect 0.5×10^6 to 1.0×10^6 CEMx174 cells for 15 h. Virus entry was compared by measuring the amount of newly synthesized viral DNA.

[b] Amounts of viral DNA represent the copy number per nanogram of total-cell DNA calculated from PCR analyses. Values in parentheses represent the relative amount of newly synthesized viral DNA in CEMx174 cells compared with the SIVmac239 results.

[c] NT, not tested.

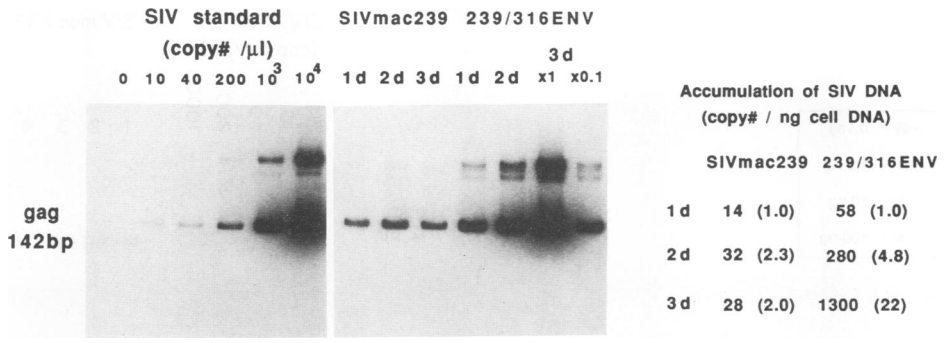

FIG. 6. Time course of SIV DNA accumulation between 1 and 3 days in macrophages. Macrophages (5×10^5) were infected with 10^5 $TCID_{50}$ of SIVmac239 or SIVmac239/316ENV (45 and 240 ng of $p27^{gag}$ antigen, respectively), and total-cell DNA was prepared 1 day (14 h), 2 days (42 h), and 3 days (66 h) postinfection. The amounts of SIV DNA were determined by PCR analyses. Amounts of SIV DNA (copy number per nanogram of cellular DNA) 1, 2, and 3 days postinfection are shown in the right panel. Relative increases of SIV DNA at 2 and 3 days are shown in parentheses.

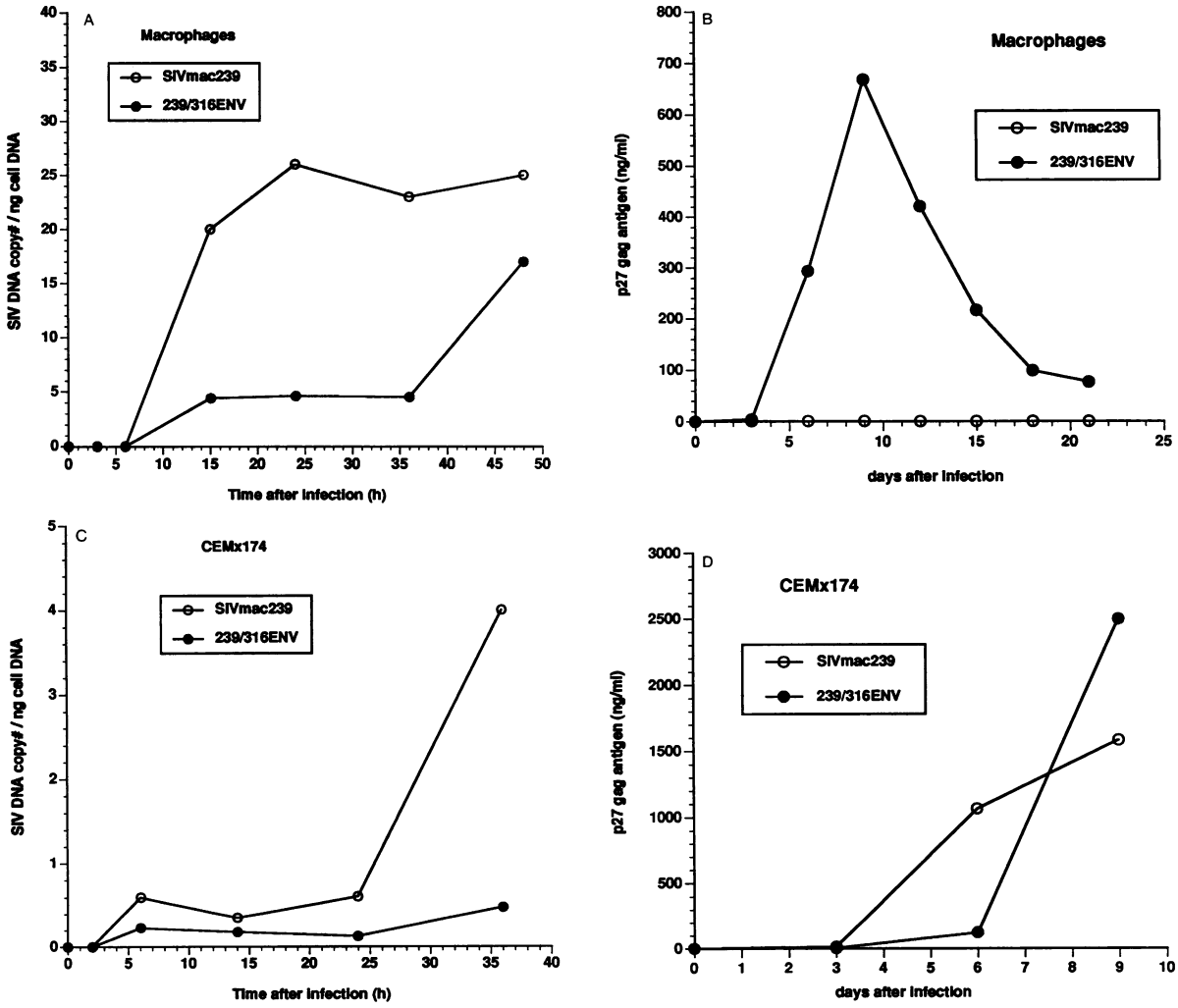

FIG. 7. Time course of SIV DNA synthesis. Macrophages or CEMx174 cells (4×10^5) were infected with SIVmac239 (3×10^4 $TCID_{50}$) or SIVmac239/316ENV (3×10^3 $TCID_{50}$) (100 and 30 ng of $p27^{gag}$ antigen, respectively). (A) Total-cell DNA from macrophages was prepared 3, 6, 15, 24, 36, and 48 h postinfection and then subjected to PCR analyses. (B) Cells taken 48 h postinfection were detached with 0.5 mM EDTA in PBS, and 25% of the cells were transferred into a 96-well plate to monitor virus production by $p27^{gag}$ antigen assay. The rest of the cells were used for PCR analyses. (C and D) Similarly, total-cell DNA from CEMx174 cells 2, 6, 14, 24, and 36 h postinfection was subjected to PCR analyses (panel C), and 25% of the cells at 36 h postinfection was used to monitor virus production (panel D).

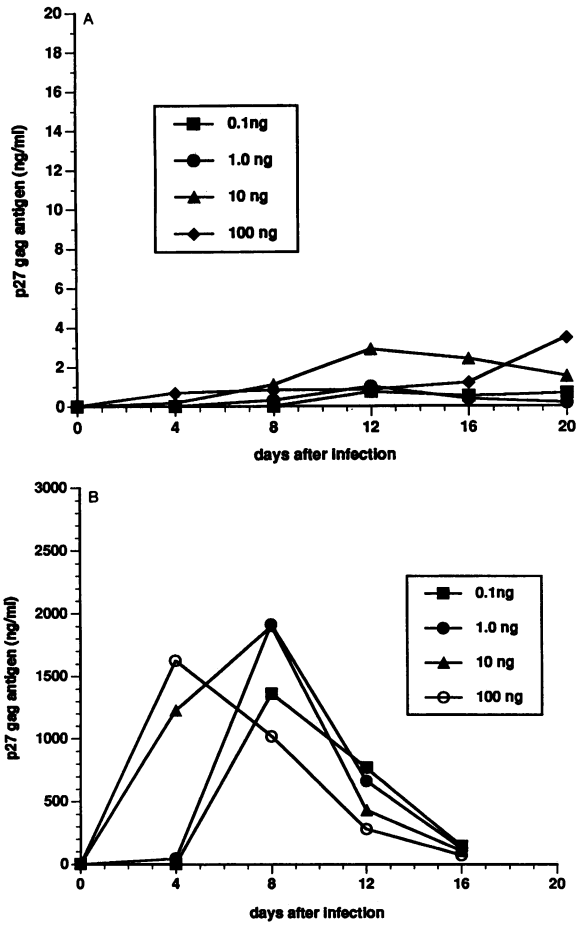

FIG. 8. Effect of inoculum dose on virus production in macrophages. Macrophages (3×10^5) were infected with SIVmac239 (A) or SIVmac239/316ENV (B) containing either 0.1, 1, 10, or 100 ng of $p27^{gag}$ antigen overnight. Virus production was monitored with a commercial $p27^{gag}$ assay kit (Coulter).

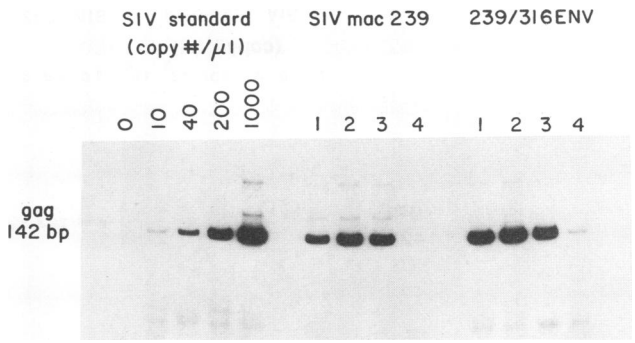

FIG. 9. Inhibition of SIVmac entry into alveolar macrophages by MAb against CD4. Macrophages (2×10^5) incubated with MAb 19thy5D7 or control MAb were infected with SIVmac239 (50 ng of $p27^{gag}$) or SIVmac239/316ENV (70 ng of $p27^{gag}$). Lanes: 1, no MAb and no preincubation with human IgG; 2, no MAb with preincubation with human IgG; 3, addition of human IgG and control MAb; 4, addition of human IgG and MAb 19thy5D7.

associated with the production of large amounts of virus (Fig. 7B). These results contrast with results obtained with CEMx174 cells, in which both viruses replicated well. The lower multiplicity of infection with SIVmac239/316ENV in CEMx174 cells resulted in lower initial levels of viral DNA (Fig. 7C) and a delay in virus production (Fig. 7D), but both viruses eventually replicated to high levels in CEMx174 cells. Thus, by adjusting the multiplicity of infection, we were able to increase the amount of SIVmac239 entering macrophages to well above that of the macrophage-tropic variant SIVmac239/316ENV, and this resulted in increased initial levels of newly reverse transcribed DNA 14 to 16 h postinfection. However, the amounts of SIVmac239 DNA in macrophages did not increase significantly after 24 h, and the ultimate result was the same: very good replication of SIVmac239/316ENV but very poor replication of SIVmac239 in macrophages.

We next investigated whether differences in replication could be overcome by very large differences in the multiplicity of infection over a 1,000-fold range. Decreasing the dosage of SIVmac239/316ENV inoculum resulted in a delay in the appearance of virus production, but in all cases high levels of SIVmac239/316ENV production were obtained (Fig. 8B). Replication of SIVmac239 was restricted to very low levels even when 100 or 1,000 times more virus was used (Fig. 8A).

Next, we investigated the dependence of SIV entry on CD4 as receptor. Since SIV infection of T lymphocytes was previously shown to be blocked with the anti-CD4 MAb 19thy5D7 (18), entry of SIVmac239 and SIVmac239/316ENV into macrophages was studied with this antibody. Entry of both SIVmac239 and SIVmac239/316ENV into macrophages was inhibited with MAb 19thy5D7 (Fig. 9, lane 4) but not control MAb (Fig. 9, lane 3).

Finally, we evaluated whether entry of SIVmac239 into cells of the macrophage cultures could be accounted for by contamination with small numbers of lymphocytes. We considered this an unlikely possibility because these cultures contained greater than 95% macrophages. Furthermore, any lymphocytes present would be unstimulated since nothing was done to activate the cells. Similar to the results of Zack et al. (49) with HIV, we have found little or no completed SIV reverse transcripts in unstimulated lymphocytes compared with stimulated lymphocytes (data not shown). Even in stimulated lymphocytes, the amounts of newly synthesized viral DNA vary in a linear fashion with the concentration of cells (data not shown). Our results indicate that entry of SIVmac239 into cells of these macrophage cultures cannot be accounted for to any appreciable extent by entry into lymphocytes.

DISCUSSION

Variants with high replicative capacity for macrophages evolved during the course of infection of some rhesus monkeys with SIVmac239 (11, 31). The emergence of these macrophage-competent variants was associated with specific pathologic manifestations, namely AIDS encephalitis and granulomatous pneumonia, in which infected macrophages constitute the pathologic lesions (11). The ability to replicate well in macrophages was shown to be due to a minimum number of changes in the envelope gene (31). Of nine amino acid changes in the envelope gene of SIVmac316, at least five were demonstrated to contribute to the maximal effect (31).

Since the envelope proteins of retroviruses are known to bind to cellular receptors and to trigger virus entry into the cell, it was quite natural to suspect that the replicative capacity of the SIVmac316 variant was determined by an

acquired ability to enter macrophages. In line with this reasoning, the inability of HIV-1_{NL4-3} to replicate well in macrophages was reported to be due to its inability to enter macrophages efficiently (35). However, our results show quite clearly that restricted replication of SIVmac239 in macrophages is not due to restricted entry into the cell. These findings appear to be analogous to results recently reported by Schmidtmayerova et al. (43), who found that HIV-1 strains LAV and NDK with poor replicative potential for macrophages entered and retrotranscribed their genomes efficiently in this cell type. The results of O'Brien et al. (35) are not necessarily in conflict with our results or those of Schmidtmayerova et al. (43); the block to replication in macrophages may occur at different stages with different strains of virus. Specific V3 sequences may be required for efficient entry into macrophages but other sequences in envelope may be required for full manifestation of the macrophage-competent phenotype.

By measuring the amounts of newly synthesized viral DNA inside the cell, we have shown that SIVmac239, which replicates poorly in macrophages, is able to enter macrophages and retrotranscribe its genomic sequences with an efficiency similar to or only slightly lower than that of envelope variants which replicate extremely well in these macrophages. Slightly reduced entry amplified many times by subsequent rounds of replication in a spreading culture cannot be the cause of the restricted replication of SIVmac239 in macrophages. Increasing the dosage of input SIVmac239 increased the amount of virus entering the cell as well as the amounts of newly synthesized DNA to levels above those seen with the macrophage-competent variants, but this was not translated into virus production above very low, basal levels. Even the use of 1,000-fold-higher levels of input virus could not overcome the block to virus production with SIVmac239 in macrophages. Thus, although restricted replication of SIVmac239 in macrophages is determined by *env*, it is not due to restricted entry.

At what step might replication be blocked in macrophages with SIVmac239? The current data allow us to conclude only that it is some step subsequent to virus entry into the cell. Late steps which require envelope, such as virus assembly, could possibly be affected. A somewhat more intriguing possibility, however, involves the response of virus expression to transcription factors or the many cytokines known to influence macrophages. A variety of factors have been shown to regulate the levels of HIV and SIV expression in macrophages (6, 30, 33, 36, 37, 46). Viral envelope proteins could conceivably be involved in the activation of or response to transcription factors, cytokines, or other regulatory signals. Such a functional role would be unusual for a retrovirus-encoded glycoprotein but perhaps not without precedent. The virus-encoded envelope glycoprotein of Friend spleen focus-forming virus (a type C retrovirus) has been found to bind to and activate the erythropoietin receptor, resulting in factor-independent cellular proliferation (26, 40).

Although SIVmac239 replication is highly restricted in macrophages, significant detectable levels of virus production do occur. Peak levels of virus antigen production following infection of macrophages with SIVmac239 generally range from 0.1 to 2.0 ng of p27 per ml. The ability of SIV and HIV to enter macrophages but to restrict their replication to very low levels could actually be a critical component of the viral life cycle in vivo. The extent to which these properties may facilitate viral persistence and dissemination in tissues is critical for understanding the pathogenesis of AIDS and other lentiviral diseases. Along these lines, it will be interesting to study the properties of these closely matched cloned viruses following experimental infection of rhesus monkeys.

ACKNOWLEDGMENTS

We thank Prabhat Sehgal for help with the lung lavages and Beverly Blake for critical comments.

This work was supported by PHS grants AI25328, AI26463, AI29855, and RR00168.

REFERENCES

1. **Armstrong, J. A., and R. Horne.** 1984. Follicular dendritic cells and virus like particles in AIDS-related lymphadenopathy. Lancet **ii**:370–372.
2. **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.
3. **Cann, A. J., J. A. Zack, A. S. Go, S. J. Arrigo, Y. Koyanagi, P. L. Green, Y. Koyanagi, S. Pang, and I. S. Y. Chen.** 1990. Human immunodeficiency virus type 1 T-cell tropism is determined by events prior to provirus formation. J. Virol. **64**:4735–4742.
4. **Chakrabarti, L., M. Guyader, M. Alizon, M. D. Daniel, R. C. Desrosiers, P. Tiollais, and P. Sonigo.** 1987. Sequence of simian immunodeficiency virus from macaque and its relationship to other human and simian retroviruses. Nature (London) **328**:543–547.
5. **Clements, J. E., and F. Wong-Staal.** 1992. Molecular biology of lentiviruses. Semin. Virol. **3**:137–146.
6. **Clouse, K. A., L. M. Consentino, K. A. Weih, S. W. Pyle, P. B. Robbins, H. D. Hochstein, V. Natarajan, and W. L. Farrar.** 1991. The HIV-I gp120 envelope protein has the intrinsic capacity to stimulate monokine secretion. J. Immunol. **147**: 2892–2901.
7. **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. **62**:4468–4476.
8. **Collman, R., N. F. Hassan, R. Walker, B. Godfrey, J. Cutilli, J. C. Hastings, H. Friedman, S. D. Douglas, and N. Nathanson.** 1989. Infection of monocyte-derived macrophages with human immunodeficiency virus type 1 (HIV-1). Monocyte-tropic and lymphocyte-tropic strains of HIV-1 show distinctive patterns of replication in a panel of cell types. J. Exp. Med. **170**:1149–1163.
9. **Cullen, B. R.** 1987. Use of eukaryotic expression technology in the functional analysis of cloned genes. Methods Enzymol. **152**:684–704.
10. **Desrosiers, R. C.** 1990. The simian immunodeficiency viruses. Annu. Rev. Immunol. **8**:557–578.
11. **Desrosiers, R. C., A. Hansen-Moosa, K. Mori, D. P. Bouvier, N. W. King, M. D. Daniel, 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.
12. **Desrosiers, R. C., and D. J. Ringler.** 1989. Use of simian immunodeficiency virus for AIDS research. Intervirology **30**: 301–312.
13. **Gardner, M. B., and P. A. Luciw.** 1988. Simian immunodeficiency viruses and their relationship to the human immunodeficiency viruses. AIDS **2**(Suppl. 1):S3–S10.
14. **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.
15. **Gendelman, H. E., J. M. Orenstein, M. A. Martin, C. Ferrua, R. Mitra, T. Phipps, L. A. Wahl, H. C. Lane, A. S. Fauci, D. S. Burke, D. Skillman, and M. S. Meltzer.** 1988. Efficient isolation and propagation of human immunodeficiency virus on recombinant colony-stimulating factor 1-treated monocytes. J. Exp. Med. **167**:1428–1441.
16. **Ho, D. D., T. R. Rota, R. T. Schooley, J. C. Kaplan, J. D. Allan,**

J. E. Groopman, L. Resnick, D. Felsenstein, C. A. Andrews, and M. S. Hirsch. 1985. Isolation of HTLV-III from cerebrospinal fluid and neural tissues of patients with neurologic syndromes related to the acquired immunodeficiency syndrome. N. Engl. J. Med. 313:1493–1497.
17. 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 cell tropism in HIV-1. Science 253:71–74.
18. Kannagi, M., J. M. Yetz, and N. L. Letvin. 1985. *In vitro* growth characteristics of simian T-lymphotropic virus type III. Proc. Natl. Acad. Sci. USA 82:7053–7057.
19. Kestler, H., T. Kodama, D. Ringler, M. Marthas, N. Pedersen, A. Lackner, D. Regier, P. Sehgal, M. Daniel, N. King, and R. Desrosiers. 1990. Induction of AIDS in rhesus monkeys by molecularly cloned simian immunodeficiency virus. Science 248:1109–1112.
20. Kestler, H. W., III, D. J. Ringler, K. Mori, D. L. Panicali, P. K. Sehgal, M. D. Daniel, and R. C. Desrosiers. 1991. Importance of the *nef* gene for maintenance of high virus loads and for development of AIDS. Cell 65:651–662.
21. Koenig, S., H. E. Gendelman, J. M. Orenstein, M. C. Dal Canto, G. H. Pezeshkpour, M. Yungbluth, F. Janotta, A. Aksamit, M. A. Martin, and A. S. Fauci. 1986. Detection of AIDS virus in macrophages in brain tissue from AIDS patients with encephalopathy. Science 233:1089–1093.
22. Koyanagi, Y., S. Miles, R. T. Mitsuyasu, J. E. Merrill, H. V. Vinters, and I. S. Y. Chen. 1987. Dual infection of the central nervous system by AIDS viruses with distinct cellular tropisms. Science 236:819–822.
23. Kwok, S., and R. Higuchi. 1989. Avoid false positives with PCR. Nature (London) 339:237–238.
24. 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.
25. Levy, J. A., J. Shimabukuro, H. Hollander, J. Mills, and L. Kaminsky. 1985. Isolation of AIDS-associated retroviruses from cerebrospinal fluid and brain of patients with neurologic symptoms. Lancet ii:586–588.
26. Li, J.-P., A. D. D'Andrea, H. F. Lodish, and D. Baltimore. 1990. Activation of cell growth by binding of Friend spleen focus-forming virus gp55 glycoprotein to the erythropoietin receptor. Nature (London) 343:762–764.
27. Lori, F., F. di Marzo Veronese, A. L. De Vico, P. Lusso, M. S. Reitz, Jr., and R. C. Gallo. 1992. Viral DNA carried by human immunodeficiency virus type 1 virions. J. Virol. 66:5067–5074.
28. 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.
29. Mitsuya, H., K. J. Weinhold, P. A. Furman, M. H. St. Clair, S. N. Lehrman, R. C. Gallo, D. Bolognesi, D. Barry, and S. Broder. 1985. 3'-Azido-3'-deoxythymidine (BW A509U): an antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus in vitro. Proc. Natl. Acad. Sci. USA 82:7096–7100.
30. Merrill, J. E., Y. Koyanagi, and I. S. Y. Chen. 1989. Interleukin 1 and tumor necrosis factor α can be induced from mononuclear phagocytes by human immunodeficiency virus type 1 binding to the CD4 receptor. J. Virol. 63:4404–4408.
31. 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.
32. Naidu, Y. M., H. W. Kestler III, Y. Li, C. V. Butler, D. P. Silva, D. K. Schmidt, C. D. Troup, P. K. Sehgal, P. Sonigo, M. D. Daniel, and R. C. Desrosiers. 1988. Characterization of infectious molecular clones of simian immunodeficiency virus (SIVmac) and human immunodeficiency virus type 2: persistent infection of rhesus monkeys with molecularly cloned SIVmac. J. Virol. 62:4691–4696.
33. Nakajima, K., O. Martinez-Maza, T. Hirano, E. C. Breen, P. G. Nishanian, J. F. Salazar-Gonzalez, J. L. Fahey, and T. Kishimoto. 1989. Induction of IL-6 (B cell stimulatory factor-2/IFN-β2) production by HIV. J. Immunol. 142:531–536.
34. Narayan, O., and J. E. Clements. 1989. Biology and pathogenesis of lentiviruses. J. Gen. Virol. 70:1617–1639.
35. 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.
36. Osborn, L., S. Kunkel, and G. J. Nabel. 1989. Tumor necrosis factor α and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor κB. Proc. Natl. Acad. Sci. USA 86:2336–2340.
37. Poli, G., P. Bressler, A. Kinter, E. Duh, W. C. Timmer, A. Rabson, J. S. Justement, S. Stanley, and A. S. Fauci. 1990. Interleukin 6 induces human immunodeficiency virus expression in infected monocytic cells alone and in synergy with tumor necrosis factor α by transcriptional and post-transcriptional mechanisms. J. Exp. Med. 172:151–158.
38. Regier, D. A., and R. C. Desrosiers. 1990. The complete nucleotide sequence of a pathogenic molecular clone of simian immunodeficiency virus. AIDS Res. Hum. Retroviruses 6:1221–1232.
39. Ringler, D. J., M. S. Wyand, D. G. Walsh, J. J. MacKey, P. K. Sehgal, M. D. Daniel, R. C. Desrosiers, and N. W. King. 1989. The productive infection of alveolar macrophages by simian immunodeficiency virus. J. Med. Primatol. 18:217–226.
40. Ruscetti, S. K., N. J. Janesch, A. Chakraborti, S. T. Sawyer, and W. D. Hankins. 1990. Friend spleen focus-forming virus induces factor independence in an erythropoietin-dependent erythroleukemia cell line. J. Virol. 64:1057–1062.
41. Sarin, P. S., Y. Taguchi, D. Sun, R. C. Gallo, and B. Oberg. 1985. Inhibition of HTLVIII/LAV replication by foscarnet. Biochem. Pharmacol. 34:4075–4079.
42. Savatier, P., G. Trabuchet, Y. Cheboune, C. Faure, G. Verdier, and V. M. Nigon. 1987. Nucleotide sequence of the beta-globin genes in gorilla and macaque: the origin of nucleotide polymorphisms in human. J. Mol. Evol. 24:309–318.
43. Schmidtmayerova, H., C. Bolmont, S. Baghdiguian, I. Hirsch, and J.-C. Chermann. 1992. Distinctive pattern of infection and replication of HIV1 strains in blood-derived macrophages. Virology 190:124–133.
44. Shioda, T., J. A. Levy, and C. Cheng-Mayer. 1991. Macrophage and T cell-line tropisms of HIV-1 are determined by specific regions of the envelope gp120 gene. Nature (London) 349:167–169.
45. Trono, D. 1992. Partial reverse transcripts in virions from human immunodeficiency and murine leukemia viruses. J. Virol. 66:4893–4900.
46. Walsh, D. G., C. J. Horvath, A. Hansen-Moosa, J. J. MacKey, P. K. Sehgal, M. D. Daniel, R. C. Desrosiers, and D. J. Ringler. 1991. Cytokine influence on simian immunodeficiency virus replication within primary macrophages. TNF-α, but not GMCSF, enhances viral replication on a per-cell basis. Am. J. Pathol. 139:877–887.
47. Watanabe, M., D. J. Ringler, M. Nakamura, P. A. DeLong, and N. L. Letvin. 1990. Simian immunodeficiency virus inhibits bone marrow hematopoietic progenitor cell growth. J. Virol. 64:656–663.
48. Westervelt, P., H. E. Gendelman, and L. Ratner. 1991. Identification of a determinant within the human immunodeficiency virus 1 surface envelope glycoprotein critical for productive infection of primary monocytes. Proc. Natl. Acad. Sci. USA 88:3097–3101.
49. Zack, J. A., S. J. Arrigo, S. R. Weitsman, A. S. Go, A. Haislip, and I. S. Y. Chen. 1990. HIV-I entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. Cell 61:213–222.
50. Zink, M. C. 1992. The pathogenesis of lentiviral disease in sheep and goats. Semin. Virol. 3:147–155.
51. Ziza, J.-M., F. Brun-Vezinet, A. Venet, C. H. Rouzioux, J. Traversat, B. Israel-Biet, F. Barre-Sinoussi, J.-C. Chermann, and P. Godeau. 1985. Lymphadenopathy-associated virus isolated from bronchoalveolar lavage fluid in AIDS-related complex with lymphoid interstitial pneumonitis. N. Engl. J. Med. 313:183.