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Isolation and Characterization of a Syncytium-Inducing, Macrophage/T-Cell Line-Tropic Human Immunodeficiency Virus Type 1 Isolate That Readily Infects Chimpanzee Cells In Vitro and In Vivo

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Fresh human immunodeficiency virus type 1 (HIV-1) isolates from patients with AIDS were screened for infectivity in chimpanzee peripheral blood mononuclear cells (PBMC) to identify strains potentially able to generate high virus loads in an inoculated animal. Only 3 of 23 isolates obtained were infectious in chimpanzee cells. Of these three, only one (HIV-1_{DH12}) was able to initiate a productive infection in PBMC samples from all 25 chimpanzees tested. HIV-1_{DH12} tissue culture infections were characterized by extremely rapid replication kinetics, profound cytopathicity, and tropism for chimp and human PBMC, primary human macrophage, and several human T-cell lines. An infection was established within 1 week of inoculating a chimpanzee with 50 50% tissue culture infective doses of HIV-1_{DH12}; cell-free virus was recovered from the plasma at weeks 1, 2, and 4 and was associated with the development of lymphadenopathy. Virus loads during the primary infection and at 6 months postinoculation were comparable to those reported in HIV-1-seropositive individuals.

There are many unanswered questions about human immunodeficiency virus type 1 (HIV-1) infections in a seropositive individual including (i) the mechanism(s) responsible for the transient depletion of CD4-positive cells that occurs immediately following exposure to the virus (for a review, see reference 48); (ii) the pathogenic process(es) leading to the gradual destruction or aberrant functioning of the immune system despite the rapid and nearly complete elimination of the virus from the blood following the acute infection (for a review, see reference 65); (iii) the irreversible loss of CD4-positive T lymphocytes that accompanies clinical progression to disease onset; (iv) the contribution, if any, of specific HIV-1 phenotypes (syncytium inducing versus non-syncytium inducing; T-cell line tropic versus macrophage tropic) to virus transmission and disease progression; and (v) the biological role of auxiliary viral genes such as *vpr*, *vif*, and *nef* during the establishment of the initial infection and its subsequent spread to other sites in the body. Because a tractable HIV-1 animal model that progresses to immunodeficiency does not exist, virtually all of our present knowledge of HIV-1 biology comes from studies of tissue culture infections or analyses of clinical specimens obtained from seropositive individuals.

The chimpanzee (*Pan troglodytes*) can be reliably infected with relatively low doses of HIV-1 (4), but no long-standing impairment to the immune system occurs (for a review, see reference 23). As with human patients, a primary HIV-1 infection of chimpanzees is usually characterized by a brief period of virus production that lasts for several weeks. Compared with human patients, however, a primary infection in chimpanzees seemed to be much milder, as suggested by an infrequent detection of plasma viremia (25, 26, 43, 46, 47). The acute infection is followed by the appearance of antibodies directed against several of the viral proteins and a rapid decline of the virus load, typically measured by the number of peripheral blood mononuclear cells (PBMC) needed for HIV-1 isolation or containing viral DNA, as determined by PCR. In many instances, virus isolation from persistently infected chimpanzees can become intermittent following resolution of the primary infection (references 4, 12, 32, 36, and 43 and our unpublished observations).

One explanation for the failure of the nearly 100 chimpanzees to progress to clinical disease following an HIV-1 challenge is that, in contrast to humans, they possess an immune system uniquely able to mount a long-lasting and protective response to HIV-1. This would be somewhat analogous to the case of sooty mangabeys (*Cercocebus atys*), the natural host of the smm strain of the simian immunodeficiency virus (SIV_{smm}), which rarely develop immunodeficiency following an SIV challenge but can experience an acute disease syndrome when inoculated with the macaque-passaged and highly pathogenic $SIV_{smmPBj14}$ (14, 24).

It is also possible that the unsuccessful attempts to develop a chimpanzee–HIV-1 disease model might be related to the intrinsic biological properties of the virus strains selected for inoculation. In the SIV-macaque system, symptoms and incubation period vary greatly among different SIV strains; several

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are not pathogenic (for a review, see reference 34). In some earlier HIV-1–chimpanzee studies, clinical specimens from patients with AIDS or primary virus isolates were used as inocula (3, 28, 29, 32, 43). It is now appreciated that most primary HIV-1 isolates do not infect chimpanzee PBMC as efficiently as human PBMC (reference 55 and this study). Among various well-characterized HIV-1 strains, only two (HIV-$1_{IIIB/LAV}$ [5] or HIV-1_{SF2} [13]) and their derivatives have been used for a majority of chimpanzee inoculations (4, 22, 26–28, 43, 47, 61); in a few instances, naive chimpanzees have been challenged with other HIV-1 strains (28, 31, 43).

In an attempt to generate an HIV-1 chimpanzee disease model with a potentially more pathogenic virus strain, primary isolates from patients with AIDS were screened for their capacity to spread with rapid kinetics and release high levels of progeny virions from chimpanzee PBMC. One of the isolates obtained, HIV-1_{DH12}, exhibited rapid infection kinetics and induced syncytia in human and chimpanzee PBMC, replicated in both human monocyte-derived macrophage (MDM) and human T-cell lines, and established an infection in a naive chimpanzee within 1 week of inoculation of 50 50% tissue culture infective doses ($TCID_{50}$) of virus. Molecular clones of HIV-1_{DH12} which directed the synthesis of virus progeny exhibiting growth properties in tissue culture infections indistinguishable from those of the parental virus were obtained.

MATERIALS AND METHODS

Cell culture. PBMC were prepared from heparinized whole blood (human and chimpanzee) or from a leukapheresis sample (human) by Ficoll-Hypaque (Pharmacia) density-gradient centrifugation. The PBMC were used immediately or preserved in cryoprotective medium (Whittaker), supplemented with 50% fetal bovine serum (FBS, HyClone) in liquid nitrogen. Fresh or frozen-thawed PBMC were stimulated with 1 μg of phytohemagglutinin (PHA, Wellcome) per ml for 3 days and then maintained in RPMI 1640 (Whittaker) medium supplemented with 10% FBS–2 mM L-glutamine (Gibco-BRL)–20 U of human recombinant interleukin 2 (Boehringer Mannheim) per ml–50 μg of gentamicin (Whittaker) per ml–100 U of penicillin-streptomycin (Gibco-BRL) per ml.

Human blood-derived monocytes were prepared as previously described (38). Briefly, elutriated monocytes were cultured in bacteriological-grade petri dishes for 2 weeks, replated in 96-well plates (Nunc no. 1-67008), and used for infection. The MDM cultures were maintained in Dulbecco's minimal essential medium (Whittaker, high-glucose, 4.5 g/liter), supplemented with 10% fresh human serum, glutamine, and antibiotics.

Human T-cell lines MT-4 (33), H9 (50), C8166 (15), and CEM-12D7 (53) were maintained in RPMI 1640 supplemented with 10% FBS, glutamine, and antibiotics. HeLa cells were maintained in Dulbecco's minimal essential medium (Whittaker) supplemented with 10% FBS, glutamine, and antibiotics.

Virus isolation from patients with AIDS. PHA-stimulated normal human PBMC (3×10^6) were cocultured with an equal number of freshly isolated PBMC from patients with AIDS. Culture supernatants were collected during a 3-week period and monitored for reverse transcriptase (RT) activity. RT-positive, cryopreserved tissue culture supernatants were used as inocula for subsequent infections.

Clinical data on the patient source of the HIV-1_{DH12} isolate. The patient was a 35-year-old male who first tested positive for HIV in July 1989 when his CD4 count was 157 cells per mm^3. From July 1990 until his last visit to the Outpatient Clinic in October 1991, he was treated with several drugs including zidovudine, didanosine, zalcitabine, and decadron. At the time of phlebotomy in October 1991, his CD4 level was 19 cells per mm^3. He died approximately 1 month later because of multiple complications of HIV infection including Kaposi's sarcoma, histoplasmosis, and toxoplasmosis.

In vitro infections. Human T-cell lines, activated human and chimpanzee PBMC (days 4 to 8 after stimulation), and human MDMs were used for in vitro virus infections. Virus inocula were normalized for ^{32}P-RT activity and used as described in the different figure legends. Culture supernatants were collected and monitored for RT activity during a 2- to 4-week period. Virion-associated RT activity was measured in the presence of [^{32}P]TTP (Amersham, >400 Ci/mmol), as previously described (62). The RT activity was reported as counts of [^{32}P]TTP per minute incorporated in 10 μl (containing 1.67 μl of infected culture supernatant) of the reaction mixture.

Virus infection of a chimpanzee. One chimpanzee (no. 1206) was pretested as negative for HIV-1 antibody (enzyme-linked immunosorbent assay [ELISA]) and HIV-1 DNA (PCR). The animal was housed in a biosafety level 2 facility, and biosafety level 3 procedures were followed. A total of 50 $TCID_{50}$ of the HIV-1_{DH12} isolate (passaged twice in human PBMC and five times in chimpanzee PBMC) was used for intravenous inoculation. The $TCID_{50}$ of HIV-1_{DH12} was determined by infecting chimpanzee PBMC in quadruplicate with serial fivefold dilutions of HIV-1_{DH12} and then assaying for RT on day 14. The animal was physically examined by veterinarians every 2 weeks, at which time blood samples were taken.

Virus isolation from blood of the infected chimpanzee. Plasma was prepared from heparinized chimpanzee blood by centrifugation ($400 \times g$) and filtration (0.45-μm-pore-size filter). PHA-stimulated chimpanzee PBMC (10^6 in 1 ml) were incubated with fivefold dilutions of plasma (starting at 1 ml of undiluted plasma) and monitored for RT production for 4 weeks. Stimulated fresh chimpanzee PBMC were added to the cultures on day 14.

PBMC, isolated from the heparinized chimpanzee blood described above, were depleted of CD8-positive cells, which can suppress virus replication in vitro and cause false-negative results for virus isolation (59). CD8 depletion was done by panning, with a CD8 antibody-coated flask (Applied Immune Science). Fivefold dilutions of CD8-depleted PBMC (starting from 5×10^6 of $CD8^-$ PBMC) were costimulated with human PBMC and monitored for RT production for 4 weeks. Stimulated human PBMC were added to the cocultures on day 14 and day 22.

Antibody ELISAs and lymphocyte immunophenotyping. Antibody in chimpanzee plasma, elicited against HIV-1 proteins, was measured by ELISA with a commercial HIV-1 micro-ELISA kit (Organon Teknika). Heparinized blood was incubated with fluorochrome-conjugated monoclonal antibodies (CD8-fluorescein isothiocyanate, CD4-phycoerythrin, CD3-peridinin chlorophyll protein [Becton Dickinson]) and then treated with fluorescence-activated cell sorter (FACS) lysing buffer (Becton Dickinson) to remove erythrocytes. Following fixation with paraformaldehyde, lymphocyte samples were analyzed by FACsort flow cytometer (Becton Dickinson).

Viral DNA copy number estimation by PCR. The copy number of viral DNA in PBMC was estimated by DNA PCR following an end-point dilution of a chimpanzee PBMC lysate. Uncultured PBMC (2×10^6) from the infected chimpanzee were lysed in 200 μl of PCR lysis buffer (100 mM KCl, 10 mM Tris-HCl [pH 8.0], 2.5 mM $MgCl_2$, 0.5% Tween 20, 0.5% Nonidet P-40, 150 μg of proteinase K per ml) for 3 h at 65°C, incubated at 100°C for 15 min, and subjected to 30 cycles of PCR. This initial PCR was carried out in duplicate with cell lysate equivalent to 10^5 cells and in quadruplicate with further fivefold dilutions of the PBMC lysate (equivalent to 2×10^4, 4,000, 800, and 160 cells). These PCR products were subsequently amplified by 30 cycles of nested PCR. Under these conditions, any amplifiable molecule would be visualized in an ethidium bromide-stained agarose gel. The distribution of amplifiable molecules in each PCR should follow the Poisson equation, $P = 1 - e^{(-m)}$, where P is the positive fraction and m is the average copy number. Since when m equals 1, P will equal 0.6321, the dilution of PBMC (starting at 10^5 cells) at which 63.21% of the samples are PCR positive would correspond to a DNA copy number of 1 per sample. The percent positive values in the PCR analyses were calculated as described by Reed and Muench (52) for determining $TCID_{50}$.

Both sets of PCR primer pairs map to the gp120 coding region of the HIV-1 *env* gene. For the first PCR, CTAAAGCCATGTGTAAAATTAACCCCACTC (located upstream of V1) and TATAGAATTCACTTCTCCAATTGTCCCT CAT (located downstream of V5) primer pairs were used. For the second amplification, TTGAAGAATGGTACTAATTTGAAGAATGG (located in the V1 region) and CCTTCAGTACCATTCCAAGTACTAT (located in the V4 region) were used. Ampli-Taq DNA polymerase (Perkin-Elmer) was used for all PCRs which were performed as follows: an initial denaturation at 94°C for 5 min; 30 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 120 s; and a final extension at 72°C for 7 min.

Molecular cloning, plasmid construction, and sequencing. The strategy followed was to clone unintegrated, circular, viral DNA molecules following their conversion to full-length linear DNA by digestion with a single cutter restriction enzyme. Earlier Southern blot analyses of HIV-1_{DH12}-infected cellular DNA revealed that *Eco*RI cleaved the viral DNA a single time within *vpr* gene sequences (data not shown).

PHA-stimulated normal chimpanzee PBMC (10^7) were infected with HIV-1_{DH12} stock at a multiplicity of infection of approximately 10^{-1}. The inoculum used had been passaged twice in human PBMC (including the primary isolation) and then three times in chimpanzee PBMC. On day 3 of infection, low-molecular-weight DNA was prepared by Hirt's method (35), digested with *Eco*RI, ligated with *Eco*RI-digested arms of Lambda Wes-B phage vector (39), and packaged in Gigapack XL (Stratagene). PCR-amplified HIV-1_{DH12} long terminal repeat (LTR), *env*, and *pol* gene fragments were labeled with [^{32}P]dCTP and used as probes for the screening of lambda phage plaques. The following oligonucleotides were used for PCR primers: GATTGGCAGAACTACACACC and TGAGGCTTAAGCAGTGGGTTC (LTR); GAACTTAATAAGAGAACTCA AG and TACAGTCTACTTGTCCATGCTA (*pol*), and TATGAATTCATATG CTGTTAAATGGCAGCAGTCTAGCAGAA and ATTTATAGAATTCTCT TCTCCATTGTCCCTCAT (*env*). Phage DNAs were purified from 7 positive plaques (out of 25,000), digested with *Eco*RI, and recloned into the T7T3-18U (Pharmacia) plasmid vector to generate a clone containing a circularly permuted HIV genome (40). *Escherichia coli* HB101 (Gibco-BRL) was used to prepare large amounts of plasmid DNA.

A plasmid carrying the two-LTR linear form of HIV-1_{DH127} was reconstructed from the original circularly permuted plasmid clone, as follows: (i) the *Xho*I (blunt ended; proviral map position 8881)-plus-*Eco*RI (position 5734) 5.9-kb cleavage fragment (containing *nef*-LTR-*gag-pol-vif* and the 5′ half of the *vpr* gene) was subcloned into the T7T3-18U vector between the *Nae*I (blunt ended) and the *Eco*RI sites; (ii) the *Eco*RI (position 5734)-plus-*Nar*I (blunt ended; proviral position 635) 4.0-kb fragment (containing the 3′ half of the *vpr* gene plus *tat-rev-vpu-env-nef* and LTR sequences) was subcloned into the pUC19 vector between the *Eco*RI and the *Hin*dIII (blunt-ended) sites; and (iii) the *Eco*RI-plus-*Bgl*I (located in the ampicillin-resistant gene of T7T3 and pUC19) cleavage products from the two subclones were ligated to one another.

Three PCR clones (DH12A, DH12B, and DH12C), containing *vpr-tat-rev-vpu-env* and *nef* sequences, were amplified from the low-molecular-weight DNA fraction of HIV-1_{DH12}-infected chimpanzee PBMC with the primer pair GCAG GAGTGGAAGCCATAA (located in *vpr*) and AAAGTCCCCAGCGGAA AGT (located in the LTR).

The nucleotide sequence of different HIV-1 clones was determined by the dideoxy-chain termination method. The complete sequence of the full-length HIV-1_{DH123} clone and the *vpu-tat-rev-env-nef*-LTR region of the HIV-1_{DH125}, HIV-1_{DH126}, and HIV-1_{DH127} clones and the *vpu-env* sequences from three PCR clones (DH12A, DH12B, and DH12C), amplified from cells infected with the uncloned HIV-1_{DH12}, were determined.

Electroporation and transfection of cloned HIV-1_{DH12} DNAs. The biological activity of the circularly permuted clones (HIV-1_{DH123}, HIV-1_{DH125}, HIV-1_{DH126}, and HIV-1_{DH127}) was evaluated by digestion of the plasmid DNA with *Eco*RI to release HIV sequences from the vector, DNA ligation to regenerate the two-LTR configuration, and electroporation of the concatemerized DNA into MT-4 cells (40). The MT-4 cells (4×10^6) were resuspended in 0.4 ml of fresh medium containing 5 μg of *Eco*RI-digested-ligated HIV-1_{DH12} DNA, electroporated at 960 μF-0.3 kV (Bio-Rad gene pulser), resuspended in 10 ml of medium containing 10^6 fresh MT-4 cells, and monitored for virus production. Samples of the infected culture supernatants were collected prior to the peak of RT activity (day 7 to day 8), filtered (0.45-μm-pore-size filter), and then used for infectivity assays.

Virus stocks were also prepared by transfecting HeLa cells with the plasmid clone carrying HIV-1_{DH127} in the two-LTR configuration (reconstructed from the circularly permuted clone as described in the previous section), HIV-1_{NL43} (1), HIV-1_{MAL} (2, 49), HIV-1_{ELI} (2, 49), HIV-1_{LAI} (49, 58), HIV-1_{AD8} (a molecular clone derived from HIV-1_{AD-87} [51]), and HIV-$1_{SG3.1}$ (31) (obtained from the National Institutes of Health AIDS Research and Reference Reagent Program). HeLa cells were transfected with 30 μg of plasmid DNA by the calcium-phosphate precipitation method. The culture supernatants were collected 48 h after transfection, filtered (0.45-μm-pore-size filter), and, following normalization for RT activity, used in infectivity assays.

RESULTS

Isolation and biological characterization of primary HIV-1 isolates capable of infecting chimpanzee PBMC. In this study, we decided to screen fresh viral isolates from patients with AIDS for their ability to induce a rapid cytopathic infection in chimpanzee PBMC, a possible predictor of pathogenicity in vivo. Prior to initiating such a search, PBMC from several uninfected chimpanzees were pretested for susceptibility to an HIV-1 isolate, previously used for chimpanzee inoculations (HIV-$1_{IIIB040}$ [4]); the cells from one animal (ISIS no. 810) reproducibly yielded high titers of virus (approximately 10^5 $TCID_{50}$/ml). A large quantity of PBMC were prepared from this chimpanzee, frozen, and used, following mitogen activation, to identify potentially useful primary HIV-1 isolates.

Attempts were made to isolate HIV-1 from 30 patients with AIDS, seen at the NIAID Outpatient Clinic, Warren Magnuson Clinical Center, National Institutes of Health, by cocultivating their PBMC with PHA-stimulated human PBMC from a seronegative individual. Virus was isolated from 23 of the 30 specimens as monitored by the release of ^{32}P-RT activity into the medium during a 21-day observation period. All 23 primary isolates produced progeny virions within 2 weeks following cell-free infection of fresh, activated human PBMC. In contrast, only 3 (HIV-1_{DH12}, HIV-1_{DH20}, and HIV-1_{DH29}) of the 23 HIV-1 isolates exhibited cell-free infectivity for chimpanzee PBMC. The other 20 samples failed to infect cells from the ISIS no. 810 animal or other chimpanzee donors.

The experiment depicted in Fig. 1 compares the infectivities of HIV-1_{DH12}, HIV-1_{DH20}, and HIV-1_{DH29} for human or chimpanzee PBMC (ISIS no. 810) with those of several other HIV-1 isolates. Three previously characterized viruses (HIV-1_{MAL} [2, 49], HIV-1_{Eli} [2, 49], and the macrophage-tropic HIV-1_{AD8} [molecularly cloned from the HIV-1_{AD-87} derivative of HIV-1_{Ada}] [30, 51]), as well as another representative (HIV-1_{DH32}) from our cohort of 30 patients with AIDS, produced no detectable progeny virus in chimpanzee PBMC. Inocula prepared from the molecularly cloned and highly tissue culture-adapted HIV-1_{Lai} (2, 49) and HIV-1_{NL43} (1) replicated to low levels in chimpanzee PBMC. The HIV-1 chimpanzee challenge stock (HIV-$1_{IIIB-040}$ [4]) and an isolate (HIV-$1_{SG3.1}$ [31]) previously shown to be tropic for chimpanzee lymphocytes readily infected chimpanzee PBMC, although their replication kinetics were slower than those of HIV-1_{DH12} and syncytium-forming activity in chimpanzee PBMC was difficult to detect (see below).

Although the data presented in Fig. 1 indicate that three of the primary HIV-1 isolates were capable of initiating infections in PBMC prepared from the ISIS no. 810 animal, the amounts of progeny virus produced differed greatly. Differences in infectivity were also observed when PBMC from other chimpanzees were monitored for susceptibility to infection by HIV-1_{DH12}, HIV-1_{DH20}, and HIV-1_{DH29}. As shown in Fig. 2, PBMC from the ISIS no. 1157 donor supported the replication of all three strains but the infection kinetics for HIV-1_{DH20} and HIV-1_{DH29} were markedly delayed compared with those for HIV-1_{DH12}. Very low levels of HIV-1_{DH20} and HIV-1_{DH29} were produced in the cells of the ISIS no. 1135 chimpanzee compared with HIV-1_{DH12}. No progeny virus was detected from the ISIS no. 1206 animal following infection of PBMC with HIV-1_{DH20} and HIV-1_{DH29}. The screening of PBMC from 21 additional chimpanzees indicated that all (25 total donors) were susceptible to HIV-1_{DH12} (Table 1), and in most cases, rapid and highly cytopathic (see below) infections were observed.

These data indicate that rapid and cytopathic infection of chimpanzee PBMC is not a common property of most HIV-1 strains, perhaps explaining the failure to detect high virus levels and clinical disease in chimpanzees inoculated with clinical specimens from patients with AIDS. No correlation between the syncytium-inducing phenotype and chimpanzee-PBMC tropism (55) was observed among HIV-1 strains in our cohort (HIV-1_{DH29} exhibited the non-syncytium-inducing phenotype, and most of our primary syncytium-inducing isolates fail to replicate in chimpanzee PBMC). Furthermore, nucleotide sequence analyses indicate that HIV-1_{DH12}, HIV-1_{DH20}, and HIV-1_{DH29} are genetically distinct clade B isolates (data not shown).

Because it was highly infectious for PBMC from all chimpanzees tested, we concentrated our efforts exclusively on the HIV-1_{DH12} isolate and initially evaluated its capacity to infect a variety of CD4-positive cell types. As shown in Fig. 3A, HIV-1_{DH12} was able to infect several different human T-cell leukemia lines although its replication kinetics in H9 and, in particular, CEM-12D7 cells were markedly delayed. It should be noted that MT-4 cells supported the production of high-titered stocks of HIV-1_{DH12} possessing the same biological properties as virus generated in PBMC. The HIV-1_{DH12} isolate also successfully infected human MDM cultures (Fig. 3B); although it exhibited characteristic rapid infection kinetics even in MDM, HIV-1_{DH12} directed the production of lower levels of progeny particles compared with a molecularly cloned virus (HIV-1_{AD8}) derived from the macrophage-tropic isolate, HIV-1_{AD-87} (51). The ability of HIV-1_{DH12} to infect both MDM and human T-cell lines was somewhat unusual but was thought to possibly reflect its recent isolation from a symptom-

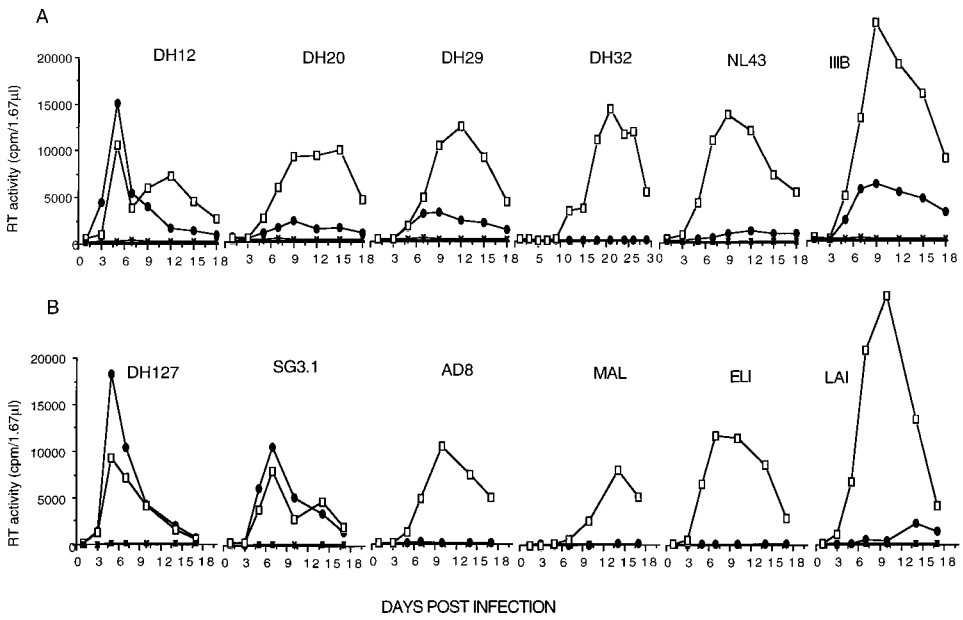

FIG. 1. (A) Infectivity of primary patient isolates and laboratory-adapted HIV-1 strains in human and chimpanzee PBMC. A total of 2.5×10^6 PHA-stimulated chimpanzee PBMC (closed circles) or human PBMC (open squares) were infected with HIV-1_{DH12}, HIV-1_{DH20}, HIV-1_{DH29}, HIV-1_{DH32}, HIV-1_{NL43} (derived from an infectious molecular clone [1]), and HIV-$1_{IIIB040}$ (4). Virus inocula were made in chimpanzee PBMC (HIV-1_{DH12}, HIV-1_{DH20}, HIV-1_{DH29}, HIV-$1_{IIIB040}$) or in human PBMC (HIV-1_{DH32}, HIV-1_{NL43}) and normalized for RT activity (approximately 10^6 ^{32}P cpm). Symbols $\times$ and + represent mock infections of human and chimpanzee PBMC, respectively. (B) Infectivity of virus derived from infectious molecular clones. A total of 10^6 PHA-stimulated chimpanzee PBMC (closed circles) or human PBMC (open squares) were infected with virus prepared from the following HIV-1 molecular clones: HIV-1_{DH127}, one of the four HIV-1_{DH12} molecular clones; HIV-$1_{SG3.1}$, a chimpanzee PBMC tropic strain (31); HIV-1_{AD8}, a macrophage-tropic, laboratory-adapted strain derived from HIV-1_{AD-87} (51); HIV-1_{MAL}, an African isolate (2, 49); HIV-1_{ELI}, an African isolate (2, 49); and HIV-1_{LAI} (49, 58), a T-cell-line-tropic laboratory strain. Virus inocula were prepared by transfecting HeLa cells and used to infect PBMC following normalization for RT activity (approximately 10^5 ^{32}P cpm). The mock infections of human ($\times$) and chimpanzee (+) PBMC, respectively, are indicated.

atic HIV-infected individual and the presence of different populations of virions with diverse tropic properties within the virus stock.

Another striking property of the HIV-1_{DH12} isolate was its capacity to rapidly induce cell fusion in PBMC, MDM, and some T-cell lines. For example, HIV-1_{DH12} induced syncytium formation in both human and chimpanzee PBMC at 3 days postinfection (chimpanzee cells are shown in Fig. 4A), whereas only aggregated cells were visualized in the two cultures following exposure to HIV-$1_{IIIB-040}$ (chimpanzee cells are shown in Fig. 4B) or HIV-$1_{SG3.1}$ (data not shown). Similarly, infection of human MDM by HIV-1_{DH12} was associated with extensive cell fusion on day 5 of the infection shown in Fig. 3B; a companion HIV-1_{AD8}-infected MDM culture contained only a few multinucleated cells on day 5. Two days later, both cultures exhibited similar cytopathicity (Fig. 4E and F). Syncytium formation was also detected following HIV-1_{DH12} infection of CEM-12D7 (Fig. 4C), C8166, and H9 cells but not MT-4 cells.

Infectivity of HIV-1_{DH12} in vivo. Since the ultimate goal of this work was to obtain a fresh HIV-1 isolate capable of rapidly initiating a de novo infection in a chimpanzee, care was taken to minimize the introduction of changes into the HIV-1_{DH12} genome during the generation of a high-titered virus inoculum. Consequently, the HIV-1_{DH12} stock used for chimpanzee inoculation was prepared by two passages in human PBMC (including its initial isolation) followed by five successive passages in chimpanzee PBMC, as described in Materials and Methods. In several independent 2-week assays, the infectivity of this inoculum was determined to be approximately 1.7×10^5 $TCID_{50}$/ml in both human and chimpanzee PBMC and about

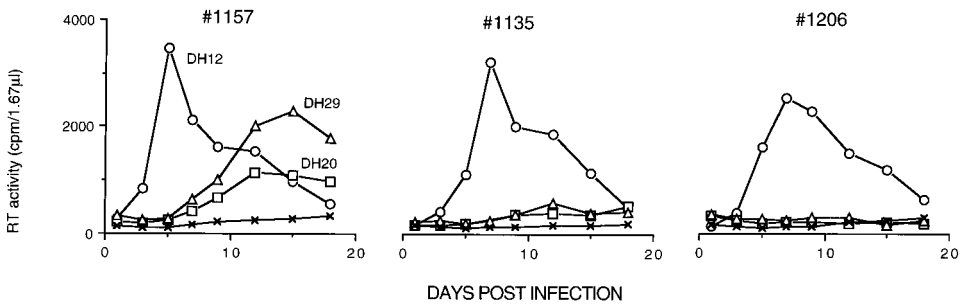

FIG. 2. Infectivity of three of the primary HIV-1 isolates in the PBMC of three different chimpanzee donors (no. 1157, no. 1135, and no. 1206). HIV-1_{DH12} (circles), HIV-1_{DH20} (squares), and HIV-1_{DH29} (triangles) inocula were prepared in chimpanzee PBMC (no. 810), normalized for RT activity (approximately 4×10^4 ^{32}P cpm), and used to infect PHA-stimulated chimpanzee PBMC (0.5×10^6 cells). Symbol $\times$ represents mock infection. The production of progeny virus was monitored by RT assay.

TABLE 1. Susceptibility of PBMC from different chimpanzee donors to primary HIV-1 isolates[a]

Virus	No. of chimpanzee donors with:	
	Susceptible PBMC	PBMC not susceptible
HIV-1_{DH12}	25	0
HIV-1_{DH20}	7	6
HIV-1_{DH29}	3	7
HIV-1_{IIIB}	9	1

[a] A total of 5×10^5 of PHA-stimulated chimpanzee PBMC were infected with cell-free virus (approximately 4×10^4 ^{32}P cpm) prepared in chimpanzee (ISIS no. 810) PBMC. Cultures were kept for at least 3 weeks, and virus replication was monitored by RT assay.

10 times higher when measured in MT-4 cells. In the animal experiment to be described, the amount of HIV-1_{DH12} inoculated is expressed as $TCID_{50}$ determined in chimpanzee PBMC.

A naive adult male chimpanzee was inoculated with 50 $TCID_{50}$ of virus by the intravenous route. As shown in Fig. 5, virus could be isolated directly from fresh plasma samples collected at weeks 1, 2, and 4 postinfection; viral RNA was also detected in the plasma at weeks 2, 3, 4, and 17 by the branched DNA procedure (reference 21 and data not shown). Although previously reported (25), the development of plasma viremia is a rarely observed phenomenon in HIV-1-inoculated chimpanzees. The establishment of an HIV-1_{DH12} infection within 1 week of inoculation was also verified by the recovery of virus following cocultivation of chimpanzee PBMC with PBMC from a seronegative human volunteer. At week 1, 5×10^6 chimpanzee cells were required for virus isolation, whereas between weeks 2 and 8, fewer (2×10^5) cells were needed, indicating the presence of increasing numbers of infected cells. Nested-DNA PCR analysis of uncultured chimpanzee PBMC, collected at various times following inoculation, was also carried out to ascertain the viral DNA copy number. HIV DNA was initially amplified (30 cycles) from serially diluted PBMC lysates, assayed in quadruplicate, with a primer pair complementary to conserved sequences in the C1 and C5 regions of gp120. These primers are able to amplify *env* gene segments from a variety of HIV-1 isolates tested including HIV-1_{Lai}, HIV-1_{NL4-3}, HIV-1_{SF2}, HIV-1_{DH12}, HIV-1_{DH20}, HIV-1_{DH29}, and four recent isolates from seropositive, asymptomatic individuals (data not shown). The second amplification (30 cycles) utilized a primer pair specific for HIV-1_{DH12} that was complementary to the V1 and V4 regions of gp120. The latter primers

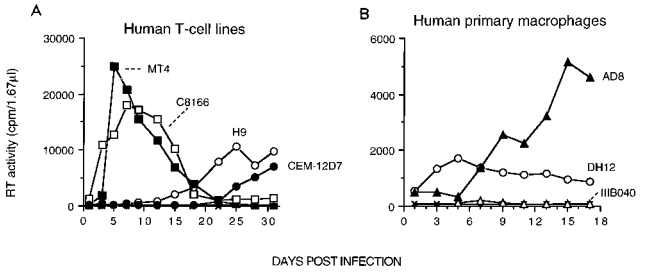

FIG. 3. HIV-1_{DH12} infects human T-cell lines (A) and MDM (B). The human T-cell lines (0.5×10^6 cells) MT-4 (33), C8166 (15), H9 (50), and CEM-12D7 (53) were inoculated with HIV-1_{DH12} (approximately 4×10^4 ^{32}P cpm), prepared in chimpanzee PBMC. Human primary macrophage cultures (10^5 cells in a 96-well plate) were inoculated with HIV-1_{DH12}, HIV-$1_{IIIB040}$, and the macrophage-tropic HIV-1_{AD8} (approximately 7×10^4 ^{32}P cpm). Mock infections (×) are also indicated.

failed to amplify the analogous region of HIV-$1_{IIIB040}$, HIV-1_{NL4-3}, HIV-1_{SF2}, HIV-1_{DH20}, and HIV-1_{DH29} (data not shown). Figure 5 indicates that the viral DNA load in PBMC peaked at weeks 3 and 8 and then fell to the 10- to 30-copy-per-10^5 PBMC range by week 15.

The infected chimpanzee developed anti-HIV-1 antibody between weeks 3 and 4 as measured by ELISA, and a sample of plasma collected at week 15 was positive at a 1:250 dilution for anti-HIV-1_{DH12} neutralizing antibody, monitored by inhibition of infectivity for human PBMC (41a). Interestingly, the infected animal also developed intermittent lymphadenopathy (weeks 3 and 18, lasting 3 to 4 weeks each time). HIV-1_{DH12} was isolated from lymphocytes prepared from the lymph node biopsied at week 3, following cocultivation with human PBMC. Although the virus load in the peripheral blood and lymph node at week 3, measured by the number of lymphocytes from each source required for virus isolation, was not significantly different, the DNA copy number per cell was approximately five times higher in the lymph node (data not shown), a result similar to that reported previously (54). The preinoculation percent CD4-positive cells (46%) fell to 33% at week 3 but by week 21 had returned to the preinfection level; no significant changes were observed in the percent CD8 cells (data not shown).

Molecular cloning of HIV-1_{DH12} DNA and characterization of virus derivatives. We noted earlier that some of the unusual biological and tropic properties of the HIV-1_{DH12} isolate might be explained by the presence of genetically diverse viruses within the uncloned HIV-1_{DH12} stock. On the other hand, these could be unique properties of the HIV-1_{DH12} isolate and not represent the contribution of discrete subpopulations of the virus stock. One way to resolve this issue would be to obtain individual molecular clones of HIV-1_{DH12} DNA and ascertain whether any directed the synthesis of virus with the tropic and syncytium-inducing properties described earlier.

Unintegrated circular viral DNA, purified from HIV-1_{DH12}-infected chimpanzee PBMC, was cleaved with *Eco*RI, cloned into the lambda phage Wes-B vector (39), and then transferred to the plasmid vector, pT7T3-18U. Of the seven clones obtained, four (DH123, DH125, DH126, and DH127) were full-length (approximately 9.2 kb in size) and circularly permuted. Their infectivity was assessed following digestion of the plasmid DNA with *Eco*RI; concatemerization in vitro using DNA ligase to regenerate the linear, two-LTR configuration; and electroporation into MT-4 cells. All four clones were able to initiate a spreading infection in MT-4 cells, releasing RT activity into the medium within a week of transfection (data not shown).

Virus stocks were prepared from the electroporated MT-4 cells and assayed for infectivity in a variety of CD4-positive cells. The virus obtained from all four infectious clones replicated in chimpanzee PBMC, human PBMC, human MDM, and human T-cell lines. Representative infections for two of the HIV-1_{DH12} molecularly cloned viruses (designated HIV-1_{DH123} and HIV-1_{DH127}) in MT-4, H9, and human primary MDM are presented in Fig. 6. Like its uncloned parent, HIV-1_{DH127} also replicated to high levels in chimpanzee PBMC as well as in human PBMC (lower left panel, Fig. 1). The cloned viruses also induced syncytia in human and chimpanzee PBMC, human MDM, and T-cell lines (HIV-1_{DH123}-infected H9 and CEM-12D7 cells are shown in Fig. 6B). Taken together, these data indicate that the unusual biological and tropic features of infections initiated by the original HIV-1_{DH12} are intrinsic properties of this isolate since they were also exhibited by molecularly cloned derivatives.

The nucleotide sequence of the entire HIV-1_{DH123} genome

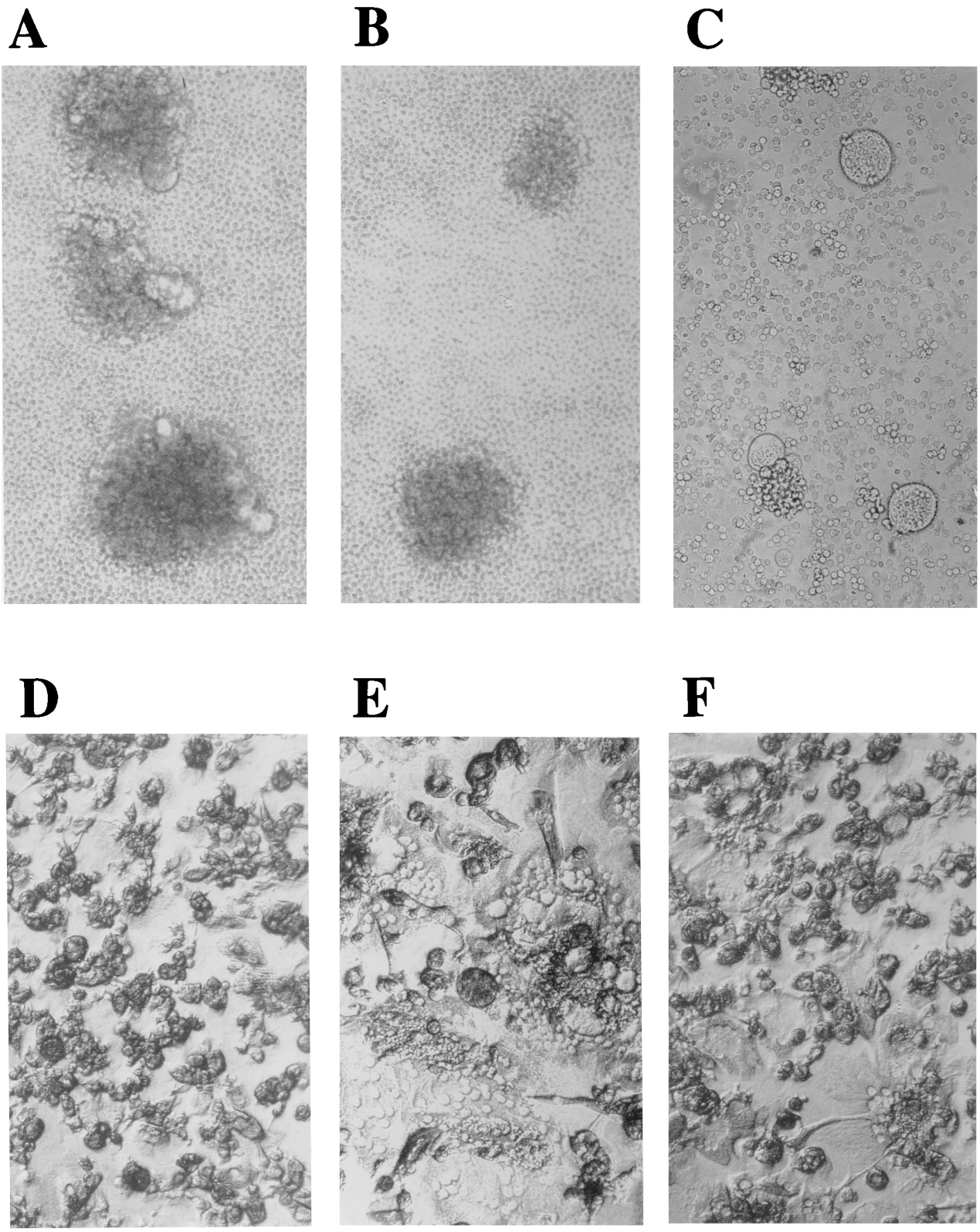

FIG. 4. Syncytium induction by HIV-1_{DH12} in various cell types. Chimpanzee PBMC, from the infection presented in Fig. 1A, are shown on day 3 following exposure to HIV-1_{DH12} (A) or HIV-$1_{IIIB040}$ (B). Syncytia were also observed in CEM-12D7 cells (0.5×10^6) 5 days following infection with an HIV-1_{DH12} inoculum (approximately 3×10^5 ^{32}P cpm of RT activity), produced in MT-4 cells (C). Uninfected (D) or HIV-1_{DH12} (E)- or HIV-1_{AD8} (F)-infected (day 7) primary human macrophage cultures are shown. The kinetics of RT production for these macrophage infections is presented in Fig. 3.

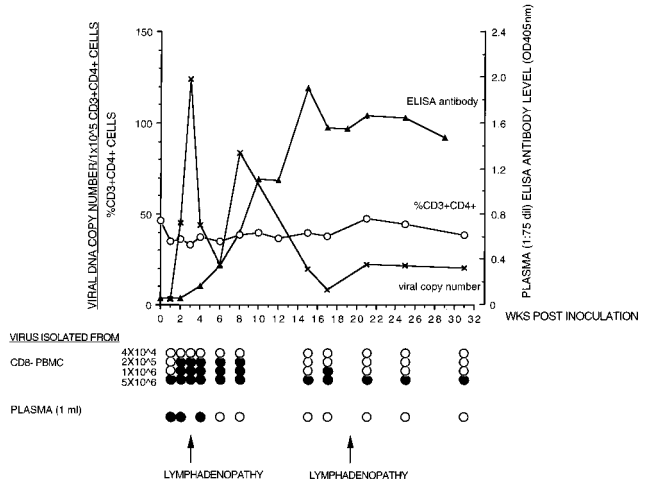

FIG. 5. Infection of a chimpanzee with HIV-1_{DH12}. Chimpanzee no. 1206 was inoculated with 50 $TCID_{50}$ of HIV-1_{DH12} at week 0. The percentage of CD3-CD4 double-positive cells in the total lymphocyte population (determined by FACS analysis), ELISA antibody levels (against HIV-1 virion proteins), and the viral copy number (estimated by quantitative DNA PCR) per 10^5 CD3-CD4 double-positive cells are indicated on the ordinates. The temporal course of virus isolation from CD8-depleted PBMC or from 1 ml of plasma is presented at the bottom (filled circles represent positive virus isolations). Two episodes of lymphadenopathy are also indicated.

as well as the 3′-terminal 4.0 kb (encompassing the *tat*, *rev*, *vpu*, *env*, and *nef* genes and 3′ LTR) of the HIV-1_{DH125}, HIV-1_{DH126}, and HIV-1_{DH127} proviral DNAs was determined. The sequence obtained placed the parental HIV-1_{DH12} strain within the clade B of HIV-1 isolates (44). As depicted diagrammatically in Fig. 7A, little heterogeneity was evident within the *env* gene sequences of the four full-length circularly permuted HIV-1_{DH12}-derived clones. Two substitutions in gp41 (both in HIV-1_{DH125}), and a valine-to-isoleucine change in the V3 loop of HIV-1_{DH127}, were the only alterations observed. No changes were present in the deduced amino acid sequences of Tat and Rev. Three additional DNA PCR clones (DH12A, DH12B, and DH12C) were obtained by amplifying the low-molecular-weight DNA from chimpanzee PBMC infected in vitro with HIV-1_{DH12}. Sequencing of the V3 loop of these latter clones was consistent with the previous analysis of the three circularly permuted full-length clones (Fig. 7B). Of particular interest, however, was the disruption of the *vpu* gene in all seven of the clones (Fig. 7B), reflecting the lack of initiation codons (DH123, DH125, DH12B), in-frame stop codons (DH126, DH12C), or frameshift mutations (DH127, DH12A).

DISCUSSION

There is still no explanation for the failure of chimpanzees to develop disease following infection with HIV-1. It is well known that the status of an animal's immune system can play a critical role in the induction of disease by retroviruses. For example, some inbred mouse strains will develop disease only if they are infected with murine leukemia viruses during the neonatal period, presumably reflecting the inability of an immature immune system to mount a protective response (6, 8, 19). Because differences have been observed during infections of human and chimpanzee cells both in vitro and in vivo, a number of immunopathological mechanisms have been proposed to explain the asymptomatic nature of HIV-1 chimpanzee infections, including (i) the reported inability of the viral gp120-chimpanzee CD4 interaction to progress to syncytium formation (9), subsequently shown to be incorrect (reference 7 and the present work); (ii) a failure of HIV-1-infected chimpanzee peripheral blood lymphocytes to undergo apoptosis following cross-linking with the CD3 monoclonal antibody (55); and (iii) the suppression of HIV-1 tissue culture infections by CD8-positive T lymphocytes, obtained from either infected or uninfected chimpanzees (11).

One could also argue that the HIV-1 strains used thus far for chimpanzee inoculations are deficient in some unknown property necessary to generate high virus loads and/or for targeting specific tissues in the infected animal. As a consequence, no disease is induced. In contrast to humans who develop high levels of both cell-free virus and infected PBMC during a primary HIV-1 infection (16), inoculated chimpanzees rarely develop a plasma viremia although infected PBMC can be detected (25, 46).

In planning a strategy for obtaining HIV-1 strains capable of replicating to high levels in a chimpanzee, we believed that the source of the isolate (a patient with AIDS) and the cell type used (chimpanzee PBMC) for preparation of the virus stock would be of critical importance. A primary isolate would more likely to contain a variety of virus subpopulations, including macrophage-tropic strains, than an isolate that had been extensively passaged in human T-cell lines. In this regard, some recent data suggest that tropism for macrophage may be of critical importance during the establishment of an HIV-1 infection in humans (42, 63, 64). Patients with AIDS were selected as the source of HIV-1 for chimpanzee inoculation in view of reports suggesting that they harbor virus strains with greater pathogenic potential than those of asymptomatic individuals (57, 60).

In addition to preserving the macrophage-tropic constituents that would be eliminated by passaging through T-leukemia cell lines, the screening of primary virus isolates for infectivity in chimpanzee PBMC as a predictor of replication potential in an inoculated animal has precedents in both the SIV and HIV systems. This approach has been useful for identifying SIV_{mac} isolates that successfully infect and induce disease in rhesus monkeys (45) or HIV-2 isolates that replicate to high titers in rhesus monkeys and baboons (10). For HIV-1, the markedly disparate amounts of virus released into the tissue culture medium from HIV-1_{IIIB} (high)- or HIV-1_{SF2} (low)-infected chimpanzee PBMC correlate with the levels of virus detected in chimpanzees subsequently inoculated with the two different virus strains (43a). Neither strain of virus induced disease, however.

Of the 23 primary isolates from patients with AIDS, only one (HIV-1_{DH12}) was able to initiate infections in all chimpanzee PBMC tested (Table 1). The prominent features of HIV-1_{DH12} infections in tissue culture include extremely rapid replication kinetics, marked cytopathicity, and tropism for a variety of $CD4^+$ cell types. The cytopathic effects associated with HIV-1_{DH12} infections may profoundly perturb cellular metabolism and, as was observed in MDM (Fig. 3B), actually reduce the amount of virus progeny released compared with the HIV-1_{Ada} isolate, which exhibits a more slowly replicating and diminished cell-killing phenotype. The dual tropism of HIV-1_{DH12} for both T-leukemia cell lines and MDM and its capacity to readily induce syncytia in PBMC were also evident in infections initiated by virus derived from molecular clones, verifying that these characteristics were, in fact, intrinsic properties of the parental uncloned HIV-1_{DH12}. Although somewhat unusual, another cloned isolate (HIV-$1_{89.6}$) with a similar extended host range has also been reported (17). No obvious

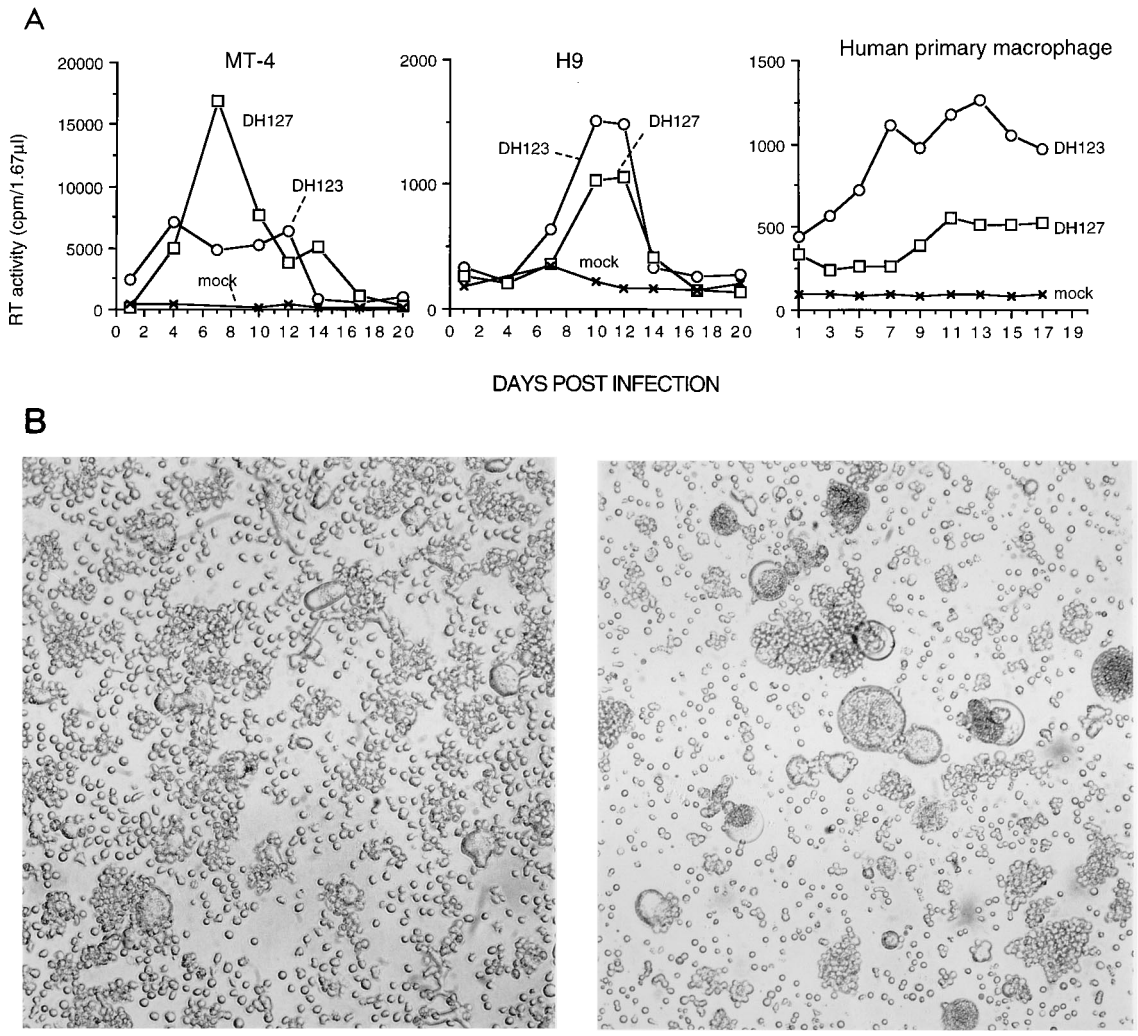

FIG. 6. Infectivity of virus derived from HIV-1_{DH12} molecular clones in a variety of CD4-positive cells. (A) The human T-cell lines (0.5×10^6 cells) MT-4 and H9 were infected with virus prepared from the HIV-1_{DH12}-derived clones DH123 and DH127 (approximately 3×10^5 ^{32}P cpm of RT activity used as inoculum). The human macrophage cultures were infected with HIV-1_{DH123} and HIV-1_{DH127} (approximately 7×10^4 ^{32}P cpm of RT activity used as inoculum). In these experiments, the virus inocula were prepared by transfecting MT-4 cells with the indicated molecular clones. (B) Syncytium formation in H9 (left) cells and 12D7 (right) cells, 5 days following infection with HIV-1_{DH123}.

amino acid homology exists between the gp120 V3 regions of HIV-1_{DH12} and HIV-$1_{89.6}$.

An interesting result of the nucleotide sequence analysis of proviral and PCR clones was the absence of a functional *vpu* gene in any HIV-1_{DH12} derivatives examined. The Vpu reading frame was altered in a number of different ways, all of which precluded the synthesis of an active protein. This same type of genetic change has been previously reported for brain-derived HIV-1 strains HIV-1_{YU-2} (41) and HIV-$1_{SG3.1}$ (31) and a derivative of the macrophage-tropic strain HIV-1_{Ada} (56a). It is of interest that the Vpu$^-$ phenotype is characterized by the accumulation of cell-associated viral proteins, impaired release of progeny virions, and increased cytopathicity during a productive virus infection (37). How and if these properties relate to the unique biological phenotype of HIV-1_{DH12} in vitro and in vivo infections are presently under investigation.

In the absence of any induced disease, comparisons of the HIV-1_{DH12} infection in inoculated chimpanzees with infection in animals with other HIV-1 isolates are somewhat problematic. The development of antibody to viral proteins and, in some instances, intermittent virus isolations have been the principal parameters monitored in HIV-1-infected chimpanzees. PCR analyses, currently used for measurements of cell-free or cell-associated virus loads in infected individuals, have been only rarely applied to HIV-1-infected chimpanzees. Nonetheless, several encouraging signs have already emerged from our HIV-1_{DH12} chimpanzee inoculations even though it is probably too early to draw any conclusions about the pathogenic potential of this isolate. A robust in vivo infection was rapidly established by inoculating chimpanzee no. 1206 with 50 $TCID_{50}$ of HIV-1_{DH12} and was associated with the presence of cell-free, cultivatable virus in the plasma at weeks 1, 2, and 4 (Fig. 5). This viremia was independently confirmed by a branched DNA signal amplification procedure. Two other naive chimpanzees have recently been inoculated with 300 and 30 $TCID_{50}$ of a high-titered stock of HIV-1_{DH12}, available for chimpanzee challenge experiments. Both became infected within 1 week, and each developed lymphadenopathy by week 3 (17a). Significantly, the two animals also developed a rash between weeks 4 and 5, which, in one chimpanzee, was widely

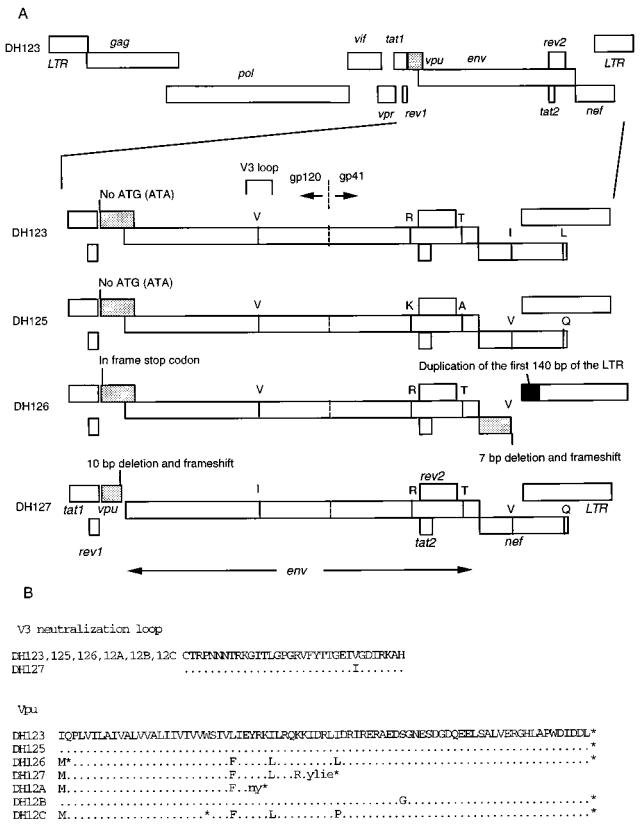

FIG. 7. Genome structure of HIV-1_{DH12}-derived clones. (A) The LTRs and open reading frames, represented by the open rectangles, of the full-length, molecularly derived, HIV-1_{DH123} isolate are shown at the top. The *tat-rev-vpu-env-nef*-LTR regions of the four infectious clones are also shown with disrupted *vpu* and/or *nef* genes, indicated by the stippled boxes. Vertical lines denote the locations of amino acid substitutions in the open reading frames. The black square in clone DH126 indicates a duplication of the first 140 bp of the U3 region. (B) A comparison of amino acid sequences comprising the gp120 V3 neutralization loop and Vpu present in HIV-1_{DH12}-derived infectious clones (DH123 and -125 to -127) or PCR clones (DH12A to -C) are shown. Dots represent amino acid identity with HIV-1_{DH123}, and asterisks indicate a termination codon. Lowercase letters signify amino acids altered because of frameshift mutations.

disseminated on the trunk, extremities, and face. Another encouraging sign is that the peak load of HIV-1_{DH12} in chimpanzee no. 1206, as measured by DNA PCR (one DNA copy per 10^3 $CD4^+$ cells), is in the same range as that reported during primary HIV-1 infections of humans (7 to 13 DNA copies per 10^3 PBMC [20]). The level present in the chimpanzee at 6 months (two copies per 10^4 $CD4^+$ cells) is also comparable to that seen in asymptomatic seropositive human patients (one copy per 6,000 to 80,000 PBMC [18, 56]). All of the HIV-1_{DH12}-infected animals are currently being monitored for additional virologic and immunologic signs suggestive of clinical progression.

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REFERENCES

1. **Adachi, A., H. E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, and M. A. Martin.** 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J. Virol. **59:**284–291.
2. **Alizon, M., S. Wain-Hobson, L. Montagnier, and P. Sonigo.** 1986. Genetic variability of the AIDS virus: nucleotide sequence analysis of two isolates from African patients. Cell **46:**63–74.
3. **Alter, H. J., J. W. Eichberg, H. Masur, W. C. Saxinger, R. Gallo, A. M. Macher, H. C. Lane, and A. S. Fauci.** 1984. Transmission of HTLV-III infection from human plasma to chimpanzees: an animal model for AIDS. Science **226:**549–552.
4. **Arthur, L. O., J. W. Bess, Jr., D. J. Waters, S. W. Pyle, J. C. Kelliher, P. L. Nara, K. Krohn, W. G. Robey, A. J. Langlois, R. C. Gallo, and P. J. Fischinger.** 1989. Challenge of chimpanzees (*Pan troglodytes*) immunized with human immunodeficiency virus envelope glycoprotein gp120. J. Virol. **63:** 5046–5053.
5. **Barre-Sinoussi, F., J. C. Chermann, F. Rey, M. T. Nugeyre, S. Chamaret, J. Gruest, C. Dauguet, C. Axler-Blin, F. Vezinet-Brun, F. Rouzioux, W. Rozenbaum, and L. Montagnier.** 1983. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science **220:**868–871.
6. **Bear, S. E., P. N. Tsichlis, and R. S. Schwartz.** 1980. H-2-mediated resistance to an ecotropic type C retrovirus: localization of controlling genes and ontogenic studies of resistance. Immunogenetics **11:**451–465.
7. **Broder, C. C., and E. A. Berger.** 1993. CD4 molecules with a diversity of mutations encompassing the CDR3 region efficiently support human immunodeficiency virus type 1 envelope glycoprotein-mediated cell fusion. J. Virol. **67:**913–926.
8. **Buller, R. S., K. Wehrly, J. L. Portis, and B. Chesebro.** 1990. Host genes conferring resistance to a central nervous system disease induced by a polytropic recombinant Friend murine retrovirus. J. Virol. **64:**493–498.
9. **Camerini, D., and B. Seed.** 1990. A CD4 domain important for HIV-mediated syncytium formation lies outside the virus binding site. Cell **60:**747–754.
10. **Castro, B. A., M. Nepomuceno, N. W. Lerche, J. W. Eichberg, and J. A. Levy.** 1991. Persistent infection of baboons and rhesus monkeys with different strains of HIV-2. Virology **184:**219–226.
11. **Castro, B. A., C. M. Walker, J. W. Eichberg, and J. A. Levy.** 1991. Suppression of human immunodeficiency virus replication by CD8+ cells from infected and uninfected chimpanzees. Cell. Immunol. **132:**246–255.
12. **Castro, B. A., C. M. Walker, M. Tateno, C. Cheng-Mayer, R. Heberling, J. W. Eichberg, and J. A. Levy.** 1989. Human immunodeficiency virus (HIV) from experimentally infected chimpanzees: isolation and characterization. J. Med. Primatol. **18:**337–342.
13. **Cheng-Mayer, C., D. Seto, M. Tateno, and J. A. Levy.** 1988. Biologic features of HIV-1 that correlate with virulence in the host. Science **240:**80–82.
14. **Cichutek, K., and S. Norley.** 1993. Lack of immune suppression in SIV-infected natural hosts. AIDS **7:**S25–S35.
15. **Clapham, P. R., R. A. Weiss, A. G. Dalgleish, M. Exley, D. Whitby, and N. Hogg.** 1987. Human immunodeficiency virus infection of monocytic and T-lymphocytic cells: receptor modulation and differentiation induced by phorbol ester. Virology **158:**44–51.
16. **Clark, S. J., M. S. Saag, W. D. Decker, S. Campbell-Hill, J. L. Roberson, P. J. Veldkamp, J. C. Kappes, B. H. Hahn, and G. M. Shaw.** 1991. High titers of cytopathic virus in plasma of patients with symptomatic primary HIV-1 infection. N. Engl. J. Med. **324:**954–960.
17. **Collman, R., J. W. Balliet, S. A. Gregory, H. Friedman, D. L. Kolson, N. Nathanson, and A. Srivivasan.** 1992. An infectious molecular clone of an unusual macrophage-tropic and highly cytopathic strain of human immunodeficiency virus type 1. J. Virol. **66:**7517–7521.

17a. **Conley, A. J. (Merck Research Laboratory).** Personal communication.

18. **Connor, R. I., H. Mohri, Y. Cao, and D. D. Ho.** 1993. Increased viral burden and cytopathicity correlate temporally with $CD4^+$ T-lymphocyte decline and clinical progression in human immunodeficiency virus type 1-infected individuals. J. Virol. **67:**1772–1777.
19. **Corbin, A., and M. Sitbon.** 1993. Protection against retroviral diseases after vaccination is conferred by interference to superinfection with attenuated murine leukemia viruses. J. Virol. **67:**5146–5152.
20. **Daar, E. S., T. Moudgil, R. D. Meyer, and D. D. Ho.** 1991. Transient high levels of viremia in patients with primary human immunodeficiency virus type 1 infection. N. Engl. J. Med. **324:**961–964.
21. **Dewar, R. L., H. C. Highbarger, M. D. Sarmiento, J. A. Todd, M. B. Vasudevachari, R. T. Davey, Jr., J. A. Kovacs, N. P. Salzman, H. C. Lane, and M. S. Urdea.** 1994. Application of branched DNA signal amplification to monitor human immunodeficiency virus type 1 burden in human plasma. J. Infect. Dis. **170:**1172–1179.
22. **Francis, D. P., P. M. Feorino, J. R. Broderson, H. M. McClure, J. P. Getchell, C. R. McGrath, B. Swenson, J. S. McDougal, E. L. Palmer, and A. Harrison.** 1984. Infection of chimpanzees with lymphadenopathy-associated

virus. Lancet **ii:**1276–1277.
23. **Fultz, P. N.** 1993. Nonhuman primate models for AIDS. Clin. Infect. Dis. **17:**S230–S235.
24. **Fultz, P. N., H. M. McClure, D. C. Anderson, and W. M. Switzer.** 1989. Identification and biologic characterization of an acutely lethal variant of simian immunodeficiency virus from sooty mangabeys (SIV/SMM). AIDS Res. Hum. Retroviruses **5:**397–409.
25. **Fultz, P. N., H. M. McClure, R. B. Swenson, and D. C. Anderson.** 1989. HIV infection of chimpanzees as a model for testing chemotherapeutics. Intervirology **30**(Suppl. 1)**:**51–58.
26. **Fultz, P. N., H. M. McClure, R. B. Swenson, C. R. McGrath, A. Brodie, J. P. Getchell, F. C. Jensen, D. C. Anderson, J. R. Broderson, and D. P. Francis.** 1986. Persistent infection of chimpanzees with human T-lymphotropic virus type III/lymphadenopathy-associated virus: a potential model for acquired immunodeficiency syndrome. J. Virol. **58:**116–124.
27. **Fultz, P. N., A. Srinivasan, C. R. Greene, D. Butler, R. B. Swenson, and H. M. McClure.** 1987. Superinfection of a chimpanzee with a second strain of human immunodeficiency virus. J. Virol. **61:**4026–4029.
28. **Gajdusek, D. C., H. L. Amyx, C. J. Gibbs, Jr., D. M. Asher, P. Rodgers-Johnson, L. G. Epstein, P. S. Sarin, R. C. Gallo, A. Maluish, L. O. Arthur, L. Montagnier, and D. Mildvan.** 1985. Infection of chimpanzees by human T-lymphotropic retroviruses in brain and other tissues from AIDS patients. Lancet **i:**55–56.
29. **Gajdusek, D. C., H. L. Amyx, C. J. Gibbs, Jr., D. M. Asher, R. T. Yanagihara, P. Rodgers-Johnson, P. W. Brown, P. S. Sarin, R. C. Gallo, A. Maluish, L. O. Arthur, R. V. Gilden, L. Montagnier, J. C. Chermann, F. B. Barre-Sinoussi, D. Mildvan, U. Mathur, and R. Leavitt.** 1984. Transmission experiments with human T-lymphotropic retroviruses and human AIDS tissue. Lancet **i:**1415–1416.
30. **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-related monocytes. J. Exp. Med. **167:**1428–1441.
31. **Ghosh, S. K., P. N. Fultz, E. Keddie, M. S. Saag, P. M. Sharp, B. H. Hahn, and G. M. Shaw.** 1993. A molecular clone of HIV-1 tropic and cytopathic for human and chimpanzee lymphocytes. Virology **194:**858–864.
32. **Gibbs, C. J., Jr., R. Peters, M. Gravell, B. K. Johnson, F. C. Jensen, D. J. Carlo, and J. Salk.** 1991. Observations after human immunodeficiency virus immunization and challenge of human immunodeficiency virus seropositive and seronegative chimpanzees. Proc. Natl. Acad. Sci. USA **88:**3348–3352.
33. **Harada, S., Y. Koyanagi, and N. Yamamoto.** 1985. Infection of HTLV-III/LAV in HTLV-1-carrying cells MT-2 and MT-4 and application in a plaque assay. Science **229:**563–566.
34. **Hirsch, V. M., and P. R. Johnson.** 1994. Pathogenic diversity of simian immunodeficiency viruses. Virus Res. **32:**183–203.
35. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26:**365–369.
36. **Johnson, B. K., G. A. Stone, M. S. Godec, D. M. Asher, D. C. Gajdusek, and C. J. Gibbs, Jr.** 1993. Long-term observations of human immunodeficiency virus-infected chimpanzees. AIDS Res. Hum. Retroviruses **9:**375–378.
37. **Klimkait, T., K. Strebel, M. D. Hoggan, M. A. Martin, and J. M. Orenstein.** 1990. The human immunodeficiency virus type 1-specific protein *vpu* is required for efficient virus maturation and release. J. Virol. **64:**621–629.
38. **Lazdins, J. K., K. Woods-Cook, M. Walker, and E. Alteri.** 1990. The lipophilic muramyl peptide MTP-PE is a potent inhibitor of HIV replication in macrophages. AIDS Res. Hum. Retroviruses **6:**1157–1161.
39. **Leder, P., D. Tiemeier, and L. Enquist.** 1977. EK2 derivatives of bacteriophage lambda useful in the cloning of DNA from higher organisms: the Lambda gtWES system. Science **196:**175–177.
40. **Leonard, J., C. Parrott, A. J. Buckler-White, W. Turner, E. K. Ross, M. A. Martin, and A. B. Rabson.** 1989. The NF-κB binding sites in the human immunodeficiency virus type 1 long terminal repeat are not required for virus infectivity. J. Virol. **63:**4919–4924.
41. **Li, Y., H. Hui, C. J. Burgess, R. W. Price, P. M. Sharp, B. H. Hahn, and G. M. Shaw.** 1992. Complete nucleotide sequence, genome organization, and biological properties of human immunodeficiency virus type 1 in vivo: evidence for limited defectiveness and complementation. J. Virol. **66:**6587–6600.
41a. **Matthews, T.** Unpublished data.
42. **McNearney, T., Z. Hornickova, R. Markham, A. Birdwell, M. Arens, A. Saah, and L. Ratner.** 1992. Relationship of human immunodeficiency virus type 1 sequence heterogeneity to stage of disease. Proc. Natl. Acad. Sci. USA **89:**10247–10251.
43. **Morrow, W. J., J. Homsy, J. W. Eichberg, J. Krowka, L. Z. Pan, I. Gaston, H. Legg, N. Lerche, J. Thomas, and J. A. Levy.** 1989. Long-term observation of baboons, rhesus monkeys, and chimpanzees inoculated with HIV and given periodic immunosuppressive treatment. AIDS Res. Hum. Retroviruses **5:**233–245.
43a. **Murthy, K. (Southwest Foundation for Biomedical Research).** Personal communication.
44. **Myers, G.** 1993. HIV-1 sequence subtypes and phylogenic trees, p. 2–3. *In* G. Myers, B. Korber, J. A. Berzofsky, and R. F. Smith (ed.), Human retroviruses and AIDS. Los Alamos National Laboratory, Los Alamos, N.Mex.
45. **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. Derosiers.** 1988. Characterization of infectious molecular clones of simian immunodeficiency virus (SIV_{mac}) and human immunodeficiency virus type 2: persistent infection of rhesus monkeys with molecularly cloned SIV_{mac}. J. Virol. **62:**4691–4696.
46. **Nara, P. L., W. Hatch, J. Kessler, J. Kelliher, and S. Carter.** 1989. The biology of human immunodeficiency virus-1 IIIB infection in the chimpanzee: in vivo and in vitro correlations. J. Med. Primatol. **18:**343–355.
47. **Nara, P. L., W. G. Robey, L. O. Arthur, D. M. Asher, A. V. Wolff, C. J. Gibbs, Jr., D. C. Gajdusek, and P. J. Fischinger.** 1987. Persistent infection of chimpanzees with human immunodeficiency virus: serological responses and properties of reisolated viruses. J. Virol. **61:**3173–3180.
48. **Niu, M. T., D. S. Stein, and S. M. Schnittman.** 1993. Primary human immunodeficiency virus type 1 infection: review of pathogenesis and early treatment intervention in humans and animal retrovirus infections. J. Infect. Dis. **168:**1490–1501.
49. **Peden, K., M. Emerman, and L. Montagnier.** 1991. Changes in growth properties on passage in tissue culture of viruses derived from infectious molecular clones of HIV-1LAI, HIV-1MAL, and HIV-1ELI. Virology **185:** 661–672.
50. **Popovic, M., M. G. Sarngadharan, E. Read, and R. C. Gallo.** 1984. Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. Science **224:**497–500.
51. **Potts, B. J., W. Maury, and M. A. Martin.** 1990. Replication of HIV-1 in primary monocyte cultures. Virology **175:**465–476.
52. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty per cent endpoints. Am. J. Hyg. **27:**493–497.
53. **Ross, E. K., A. J. Buckler-White, A. B. Rabson, G. Englund, and M. A. Martin.** 1991. Contribution of NF-κB and Sp1 binding motifs to the replicative capacity of human immunodeficiency virus type 1: distinct patterns of viral growth are determined by T-cell types. J. Virol. **65:**4350–4358.
54. **Saksela, K., E. Muchmore, M. Girald, P. Fultz, and D. Baltimore.** 1993. High viral load in lymph nodes and latent human immunodeficiency virus (HIV) in peripheral blood cells of HIV-1-infected chimpanzees. J. Virol. **67:**7423–7427.
55. **Schuitemaker, H., L. Meyaard, N. A. Kootstra, R. Dubbes, S. A. Otto, M. Tersmette, J. L. Heeney, and F. Miedema.** 1993. Lack of T cell dysfunction and programmed cell death in human immunodeficiency virus type 1-infected chimpanzees correlates with absence of monocytotropic variants. J. Infect. Dis. **168:**1140–1147.
56. **Simmonds, P., P. Balfe, J. F. Peutherer, C. A. Ludlam, J. O. Bishop, and A. J. Brown.** 1990. Human immunodeficiency virus-infected individuals contain provirus in small numbers of peripheral mononuclear cells and at low copy numbers. J. Virol. **64:**864–872.
56a. **Theodore, T. S., et al.** Unpublished data.
57. **van Griensven, G. J., E. M. de Vroome, F. de Wolf, J. Goudsmit, M. Roos, and R. A. Coutinho.** 1990. Risk factors for progression of human immunodeficiency virus (HIV) infection among seroconverted and seropositive homosexual men. Am. J. Epidemiol. **132:**203–210.
58. **Wain-Hobson, S., P. Sonigo, O. Danos, S. Cole, and M. Alizon.** 1985. Nucleotide sequence of the AIDS virus, LAV. Cell **40:**9–17.
59. **Walker, C. M., D. J. Moody, D. P. Stites, and J. A. Levy.** 1986. CD8+ lymphocytes can control HIV infection in vitro by suppressing virus replication. Science **234:**1563–1566.
60. **Ward, J. W., T. J. Bush, H. A. Perkins, L. E. Lieb, J. R. Allen, D. Goldfinger, S. M. Samson, S. H. Pepkowitz, L. P. Fernando, P. V. Holland, S. H. Kleinman, A. J. Grindon, J. L. Garner, G. W. Rutherford, and S. D. Holmberg.** 1989. The natural history of transfusion-associated infection with human immunodeficiency virus. Factors influencing the rate of progression to disease. N. Engl. J. Med. **321:**947–952.
61. **Watanabe, M., D. J. Ringler, P. N. Fultz, J. J. MacKey, J. E. Boyson, C. G. Levine, and N. L. Letvin.** 1991. A chimpanzee-passaged human immunodeficiency virus isolate is cytopathic for chimpanzee cells but does not induce disease. J. Virol. **65:**3344–3348.
62. **Willey, R. L., D. Smith, L. Lasky, T. S. Theodore, P. L. Earl, B. Moss, D. J. Capon, and M. A. Martin.** 1988. In vitro mutagenesis identifies a region within the envelope gene of the human immunodeficiency virus that is critical for infectivity. J. Virol. **62:**139–147.
63. **Zhang, L. Q., P. MacKenzie, A. Cleland, E. C. Holmes, A. J. Brown, and P. Simmonds.** 1993. Selection for specific sequences in the external envelope protein of human immunodeficiency virus type 1 upon primary infection. J. Virol. **67:**3345–3356.
64. **Zhu, T., H. Mo, N. Wang, D. S. Nam, Y. Cao, R. A. Koup, and D. D. Ho.** 1993. Genotypic and phenotypic characterization of HIV-1 patients with primary infection. Science **261:**1179–1181.
65. **Zunich, K. M., and H. C. Lane.** 1991. Immunologic abnormalities in HIV infection. Hematol. Oncol. Clin. N. Am. **5:**215–228.