

NOTES

Rapid CD4⁺ T-Cell Loss Induced by Human Immunodeficiency Virus Type 1_{NC} in Uninfected and Previously Infected Chimpanzees

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To investigate the pathogenicity of a virus originating in a chimpanzee with AIDS (C499), two chimpanzees were inoculated with a plasma-derived isolate termed human immunodeficiency virus type 1_{NC} (HIV-1_{NC}). A previously uninfected chimpanzee, C534, experienced rapid peripheral CD4⁺ T-cell loss to fewer than 26 cells/ μ l by 14 weeks after infection. CD4⁺ T-cell depletion was associated with high plasma HIV-1 loads but a low virus burden in the peripheral lymph node. The second chimpanzee, C459, infected 13 years previously with HIV-1_{LAV}, experienced a more protracted course of peripheral CD4⁺ T-cell loss after HIV-1_{NC} inoculation, resulting in fewer than 200 cells/ μ l by 96 weeks postinoculation. The quantities of viral RNA in the plasma and peripheral lymph node from C459 were below the lower limits of detection prior to inoculation with HIV-1_{NC} but were significantly and persistently increased after superinfection, with HIV-1_{NC} representing the predominant viral genotype. These results show that viruses derived from C499 are more pathogenic for chimpanzees than any other HIV-1 isolates described to date.

The development of AIDS in individuals infected with human immunodeficiency virus type 1 (HIV-1) is most often characterized by a chronic depletion of CD4⁺ T cells. The destruction of CD4⁺ T cells results in the development of severe immunodeficiency, which leads to the emergence of opportunistic infections and neoplasia, ultimately culminating in death (9). Despite the development of a number of animal models of pathogenic lentiviral infection, including simian immunodeficiency virus (SIV) (2, 13, 25) and HIV-2 infection of macaques (22, 28), and the development of pathogenic HIV-SIV chimeric viruses (21, 31), an animal model of pathogenic HIV-1 infection has not been developed.

Experimental infection of chimpanzees with HIV-1 was first demonstrated in 1984, soon after the discovery of the virus (1, 11). Subsequently, a large number of chimpanzees have been experimentally inoculated with various isolates of HIV-1 as part of HIV pathogenesis investigations or vaccine technology research. Most HIV-1 isolates have been shown to be non-pathogenic in chimpanzees; thus, the relevance of vaccine protection in this model has been the subject of considerable controversy. The absence of disease development in HIV-1-infected chimpanzees has stimulated investigations aimed at understanding the mechanisms responsible for apathogenic

lentivirus infection (4, 7, 8, 10, 17–19, 27, 32, 37). However, no conclusions have been drawn regarding a definitive mechanism of resistance to disease development.

To date, only one chimpanzee infected with HIV-1 has developed AIDS (29). That animal, C499, was euthanized as a result of severe CD4⁺ T-cell loss, thrombocytopenia, and chronic diarrhea due to *Cryptosporidium* infection. Transfusion of blood from C499 to an HIV-1-negative chimpanzee, C455, resulted in rapid CD4⁺ T-cell loss in the latter animal (29). This observation suggested that a chimpanzee-pathogenic strain of HIV had evolved in C499, and subsequent genetic analyses of viruses isolated from C499 and C455 showed that the virulent strain was likely a recombinant of HIV-1_{LAV1b} and HIV-1_{SF2} (26). To confirm that virus from C499 is pathogenic for chimpanzees, we inoculated two additional chimpanzees with a virus isolated from the plasma of C455. (The Yerkes Regional Primate Research Center is fully accredited by the American Association for the Accreditation of Laboratory Animal Care, and all animals were housed in accordance with Animal Welfare Act guidelines.) The results presented here conclusively demonstrate the *in vivo* evolution of an HIV-1 variant that is pathogenic for chimpanzees.

The HIV-1_{NC} isolate, derived from the plasma of chimpanzee C455, has been described previously (26). It was grown in chimpanzee peripheral blood mononuclear cells (PBMC) and, at peak reverse transcriptase activity, cell-free supernatant was harvested, aliquoted, and stored under liquid nitrogen. This virus stock had a titer of 10⁴ 50% tissue culture infective doses

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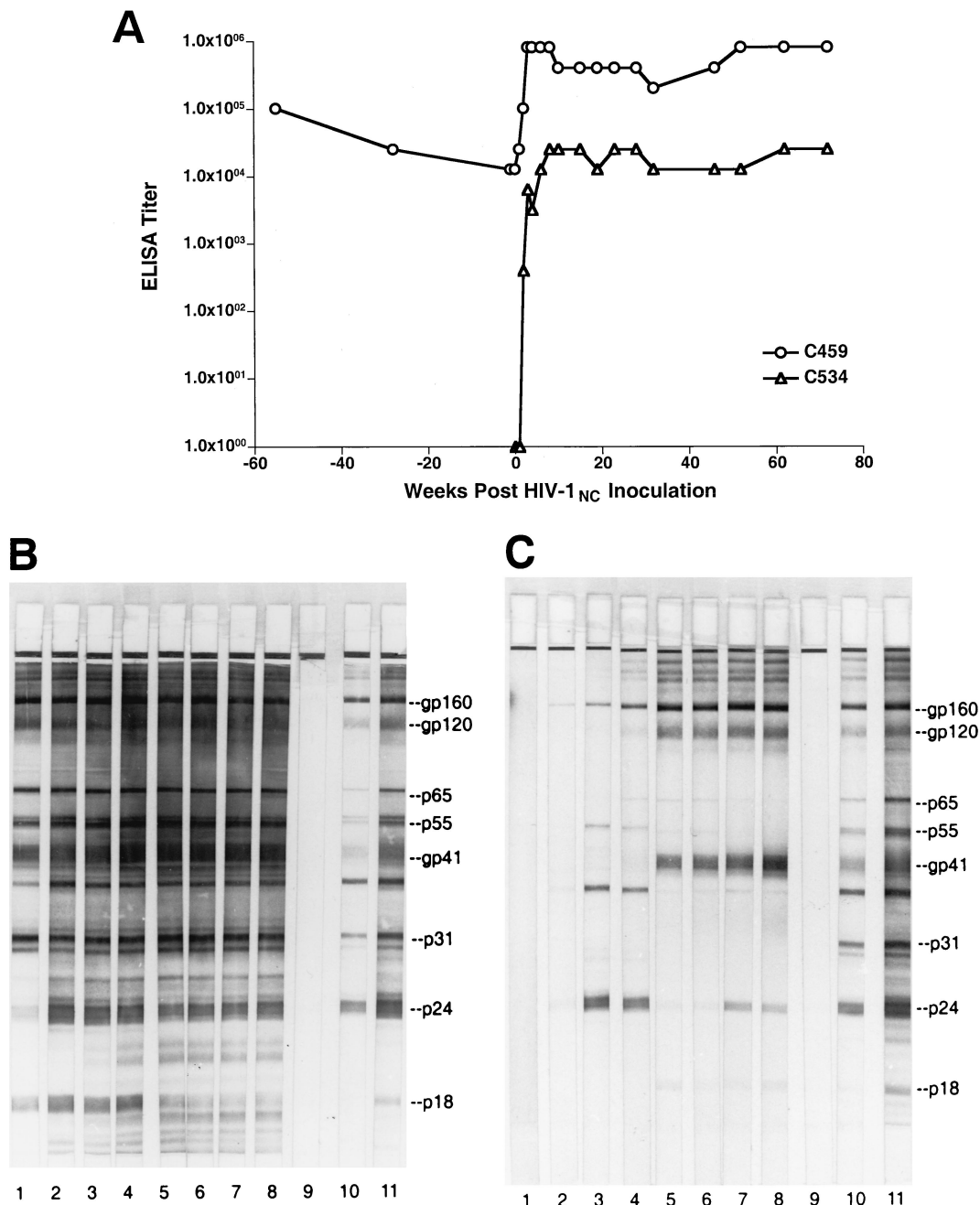


FIG. 1. HIV-specific antibody responses following HIV-1_{NC} infection in chimpanzees. (A) Plasma samples obtained from C459 and C534 at the indicated times postinfection were used to quantify HIV-specific antibody responses using a commercially available whole-virus ELISA. (B and C) Qualitative analyses of viral antibody were performed using commercially available Western blot strips. In panel B, plasma samples were from animal C459; in panel C, plasma samples from animal C534. Lane 1, day of inoculation; lane 2, 2 weeks postinoculation; lane 3, 8 weeks postinoculation; lane 4, 15 weeks postinoculation; lane 5, 32 weeks postinoculation; lane 6, 53 weeks postinoculation; lane 7, 63 weeks postinoculation; lane 8, 80 weeks postinoculation; lane 9, plasma from an uninfected chimpanzee; lane 10, weak positive control serum; lane 11, strong positive control serum.

(TCID₅₀)/ml and had an HIV-1 p24 antigen concentration of 295.8 ng/ml. Two chimpanzees, C459 and C534, were intravenously inoculated with a 10⁴ TCID₅₀ of HIV-1_{NC}. C534 was a naive animal; however, C459 was HIV positive, having been previously infected with the LAV isolate of HIV-1 (HIV-1_{LAV}) in 1984 (14). Prior to infection with HIV-1_{NC}, C459 had a

vigorous antibody response to infection with HIV-1_{LAV} as measured in plasma by enzyme-linked immunosorbent assay (ELISA) (Fig. 1A) and Western blotting (Fig. 1B). After superinfection, C459 showed a dramatic increase in HIV-1-specific antibody, from a titer of 1:12,800 on the day of HIV-1_{NC} inoculation to a titer of 1:819,000 at 3 weeks postinfection.

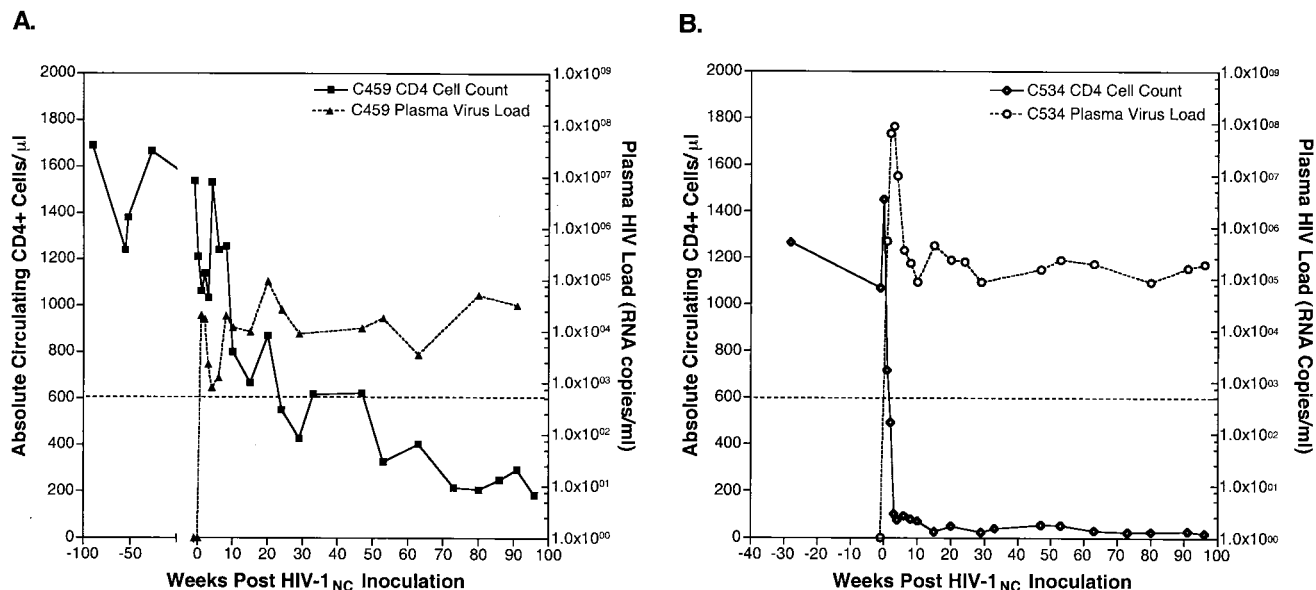


FIG. 2. Peripheral CD4⁺ T-cell levels and plasma HIV loads in HIV-1_{NC}-infected chimpanzees. At the indicated times pre- and post-HIV-1_{NC} inoculation, blood was obtained from chimpanzees C459 (A) and C534 (B) for use in determination of circulating levels of CD4⁺ CD2⁺ T cells by flow cytometry and for use in the quantitation of plasma viral RNA levels. The dotted line represents the cutoff level for the quantitation of plasma HIV levels (800 copies/ml).

Western blot analysis using commercially available HIV-1 strips (Cambridge Biotech, Rockville, Md.) (Fig. 1B) revealed that increases in reactivity to HIV-1-specific proteins were primarily directed against the p24 and p18 capsid antigens following superinfection with HIV-1_{NC}.

In the previously uninfected animal, C534, a rapid antibody response to HIV-1 developed following inoculation with the NC isolate. As measured by ELISA, HIV-1-specific antibody titers rose rapidly and stabilized at 1:25,600 by 8 weeks postinoculation (Fig. 1A). While humoral responses of this magnitude are not typically observed during primary infection of chimpanzees with tissue culture-adapted viruses such as HIV-1_{LAV} and HIV-1_{SF2}, responses of this intensity are observed in macaques infected with highly pathogenic isolates of SIV (5, 24, 34). Western blot analysis showed that the humoral response generated by C534 during acute infection was directed primarily against Gag proteins p24 and p55, with lesser reactivity toward Env gp160 (Fig. 1C). At 32 weeks postinfection, a decline in p24 reactivity was observed, with an increase in Env gp41 and gp120 reactivities. As shown, the peak antibody titer observed for C534 did not approach that of C459.

Virus could be isolated from PBMC collected from both C459 and C534 at multiple times following inoculation (data not shown). The quantity of HIV-1 RNA was measured in plasma specimens from both chimpanzees throughout the course of HIV-1_{NC} infection by quantitative competitive PCR as described previously (29). The quantity of viral RNA in previously infected chimpanzee C459 was below the lower limit of detection (800 copies/ml) prior to superinfection (Fig. 2A). At 1 week post-HIV-1_{NC} inoculation, C459 had a plasma virus load of 2×10^4 RNA copies/ml. The plasma virus load in this animal peaked at 19 weeks postinfection (9×10^4 copies/ml). Subsequently, the viral setpoint for this animal has been main-

tained at between 3×10^3 and 6×10^4 RNA copies/ml. For C534, plasma virus load peaked by 3 weeks postinfection at 8.8×10^7 RNA copies/ml and has subsequently achieved equilibrium between 2×10^4 and 2×10^5 RNA copies/ml (Fig. 2B).

The absolute number of peripheral CD3⁺/CD4⁺ T lymphocytes was measured in whole blood specimens from both chimpanzees following HIV-1_{NC} inoculation. CD4⁺ T cells decreased during the first weeks after HIV-1_{NC} inoculation of C459 but remained within normal limits (Fig. 2A). After rebounding to 1,500 cells/ μ l by 4 weeks postinoculation, the number of CD4⁺ T cells steadily decreased to 196 cells/ μ l by 96 weeks post-HIV-1_{NC} inoculation. In contrast to the slowly progressive depletion observed for C459, immediate and sustained CD4⁺ T-cell loss was observed after infection of C534. For C534, the number of CD4⁺ T cells decreased by 50% to 718 cells/ μ l by 1 week postinoculation and further decreased to fewer than 100 cells/ μ l by 4 weeks postinoculation, where they remained for the balance of the study (96 weeks postinoculation).

Lymph node biopsies were obtained from C459 and C534 prior to and at 3 weeks after inoculation with HIV-1_{NC}. Lymph nodes from both animals prior to HIV-1_{NC} inoculation showed normal numbers of lymphocytes and little to no secondary follicle formation, similar to other age-matched HIV-negative and HIV-infected nonprogressor chimpanzees (30). The number and distribution of productively infected cells was determined in sections of lymph node by in situ hybridization for viral RNA, using a riboprobe that spans the entire HIV-1 genome, as described elsewhere (29, 30). Virus-infected cells were not observed in sections of lymph node collected from C459 prior to HIV-1_{NC} infection (Fig. 3A). This finding is in agreement with the low virus burden observed in the plasma of C459 on the day of biopsy and is consistent with observations

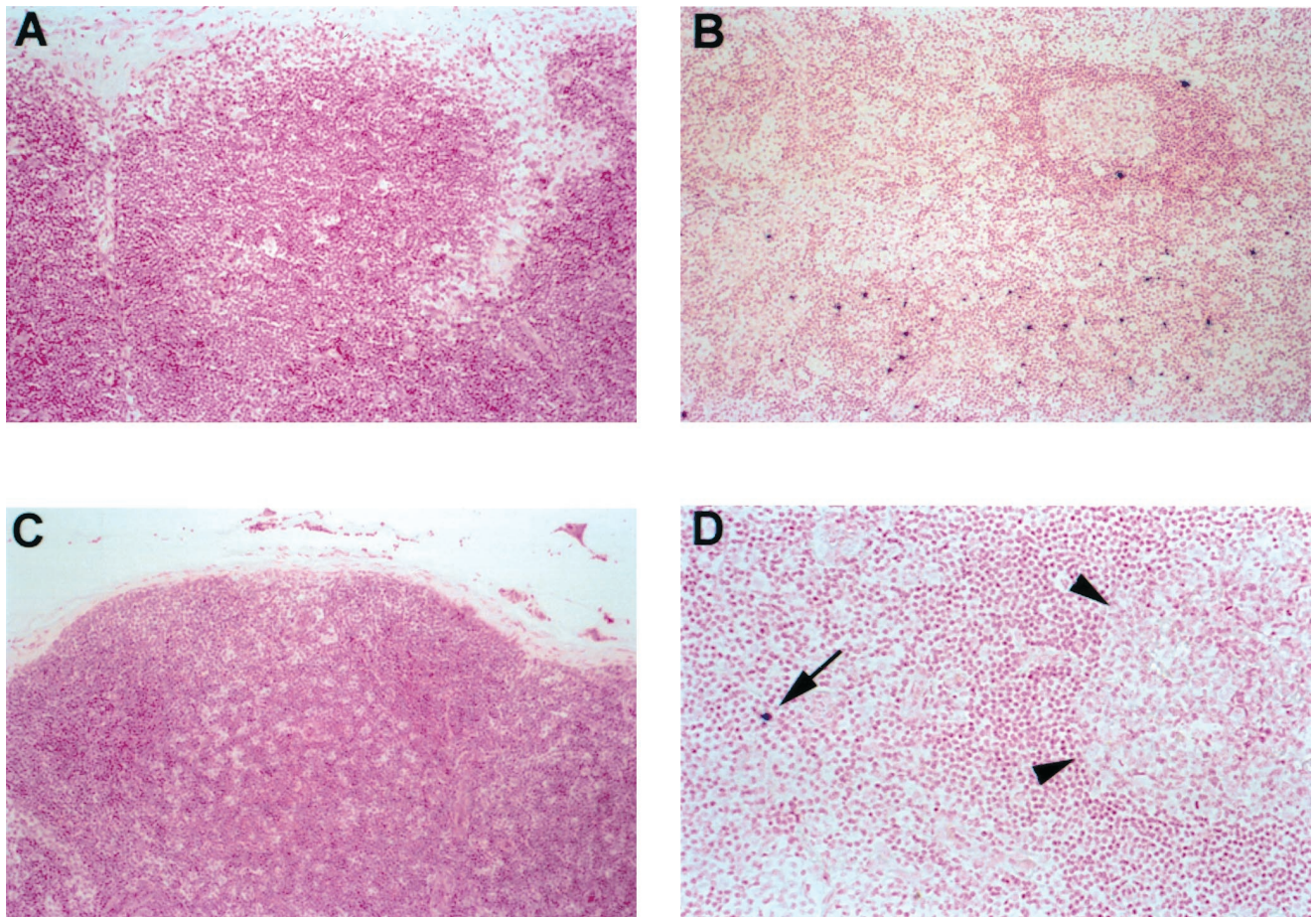


FIG. 3. Distribution of productively infected cells in lymph nodes from HIV-1_{NC}-infected chimpanzees. In situ hybridization for HIV-1 RNA is shown for chimpanzees C459 (A and B) and C534 (C and D), using the chromagen nitroblue tetrazolium-BCIP (5-bromo-4-chloro-3-indolylphosphate) and nuclear fast red counterstain. (A) Productively infected cells are not apparent in sections of lymph node from HIV-1 infected chimpanzee C459 prior to inoculation with HIV-1_{NC} ($\times 100$). (B) Numerous infected cells (dark blue stain) are seen in the paracortex of a lymph node collected from C459 3 weeks after superinfection with HIV-1_{NC} ($\times 100$). (C) Lymph node from C534 (uninfected chimpanzee) prior to infection with HIV-1_{NC} ($\times 100$). (D) HIV-1-infected cell (arrow) shown in the paracortex of a lymph node from C534 3 weeks after infection with HIV-1_{NC}. Arrowheads delineate the margins of a germinal center ($\times 200$). Note the absence of secondary follicles in lymph nodes from HIV-infected, nonprogressor C459 (A) and HIV-negative C534 (C).

made in other HIV-infected nonprogressor chimpanzees in the Yerkes cohort (30). Moderate numbers of infected cells were localized within germinal centers and paracortical regions of the lymph node specimen collected from C459 3 weeks after superinfection with HIV-1_{NC} (Fig. 3B). Surprisingly, only small numbers of infected cells were observed in sections of lymph node collected from C534 at 3 weeks after infection with HIV-1_{NC}, despite an extremely high plasma virus load measured on the day of biopsy (Fig. 3D). As with C459, infected cells were located primarily within the paracortical regions of the node. In addition, we detected a faint intercellular hybridization signal within the germinal centers, suggesting dendritic cell trapping of immune-complexed virions (data not shown).

The sudden increase in plasma and lymphoid virus burdens and progressive CD4⁺ T-cell depletion observed following superinfection of C459 suggested that HIV-1_{NC} had initiated a pathogenic infection in the face of an apathogenic infection with HIV-1_{LAV}. To confirm the identity of the predominant viral genotype present after HIV-1_{NC} inoculation, we analyzed

the genotype of virus present in C459 prior to and after inoculation with HIV-1_{NC}. As mentioned above, previous studies indicated that HIV-1_{NC} was a recombinant virus, one derived from both HIV-1_{LAV1b} and HIV-1_{SF2}. Regions of identifiable recombination included both *gag* and *vpr* (26). One region in the *gag* of HIV-1_{NC} specifically resembled HIV-1_{SF2}, containing a 36-bp deletion relative to HIV-1_{LAV}. A second site, located in *vpr*, also resembled HIV-1_{SF2}, containing a 3-bp insertion relative to HIV-1_{LAV}. These sites were utilized as molecular markers for genotyping virus from C459. Genomic DNA prepared from the PBMC of C459 was utilized to amplify both *gag* and *vpr* regions of the HIV-1 genome using typical single-round PCR (High Fidelity Kit; Boehringer Mannheim). The primers used for the amplification of the *gag* gene were as follows: forward, 5'-TGGCAAAGAAGGGCACATAG-3'; reverse, 5'-TGAGGGAAGTTAAAGGATACAGTT-3' (these primers amplify a region of *gag* that is 320 bp long in HIV-1_{LAV} and 284 bp long in HIV-1_{SF2}); the genetic coordinates in HIV-1_{LAV} are 1520 to 1839). The primers used for amplification of

A.

Gene	Virus	Nucleotide Sequence
gag	HIV-1 _{LAV}	ccaacagccccaccatttcttcagagcagaccagagccaacagccccaccagaagagag
	HIV-1 _{SF2}	ccaacagccccacca-----gaagagag
	HIV-1 _{JC/NC}	ccaacagccccacca-----gaagagag
vpr	HIV-1 _{LAV}	agcagaataggcgttactcaacagaggagagca---agaaatggagccagtagatcc
	HIV-1 _{SF2}	agcagaataggcgttactcaacagaggagagcaagaagaaatggagccagtagatcc
	HIV-1 _{JC/NC}	agcagaataggcgttactcaacagaggagagcaagaagaaatggagccagtagatcc

B.

Sample Date	Weeks Pre/Post Infection	Gene	Virus Homology	No. analyzed/No. identical
11/19/96	26-Pre	gag	HIV-1 _{LAV}	20/20
		vpr	HIV-1 _{LAV}	20/20
9/9/97	15-Post	gag	HIV-1 _{SF2/JC/NC}	20/20
		vpr	HIV-1 _{SF2/JC/NC}	20/20

FIG. 4. Nucleotide sequence analysis of HIV-1 genes in chimpanzee C459. (A) Sequence alignments of portions of *gag* and *vpr* genes of HIV-1_{LAV}, HIV-1_{SF2}, and HIV-1_{JC/NC}, illustrating major differences. (B) PBMC from chimpanzee C459 obtained pre- and post-HIV-1_{NC} infection (sample dates 11/19/96 and 9/9/97, respectively) were used to prepare genomic DNA for use as template in PCR experiments. Amplified DNA was cloned into the pGEM-T vector. Twenty clones obtained from each sample were analyzed by DNA sequencing. Results show that, prior to HIV-1_{NC} infection, sequences were most similar to HIV-1_{LAV} (the virus used to inoculate animal C459 in 1984) but that, after HIV-1_{NC} inoculation, sequences were most similar to HIV-1_{SF2} and HIV-1_{JC/NC} (26).

the *vpr* gene were as follows: forward, 5'-ATGGAACAAGC CCCAGAAGACCAAGGG-3'; reverse, 5'-GGATCTACTGG CTCCAGGCTCT-3' (these primers amplify a region of *vpr* that is 288 bp long in HIV-1_{LAV} and 291 bp long in HIV-1_{SF2}; the genetic coordinates in HIV-1_{LAV} are 5141 to 5428). Twenty clones were analyzed for each time point. Six months prior to inoculation with HIV-1_{NC}, the viral genotype detected in the PBMC of C459 was most like LAV in both *gag* and *vpr* (Fig. 4). However, at 15 weeks after superinfection, the predominating virus was SF2-like in *gag* and *vpr*, indicating that the replicating virus was the superinfecting HIV-1_{NC}.

Despite the fact that most HIV-1 isolates have been shown to produce nonpathogenic infections in chimpanzees, continuing efforts have been made to find or derive an isolate of HIV-1 that is more pathogenic for this species (3, 16, 33, 34). Recent findings suggest that chimpanzees are a natural reservoir for SIVcpz and may have served as the source of HIV-1 introduction into the human population (15). Host adaptation to primate lentiviruses most likely explains why experimental infection of chimpanzees with HIV usually results in apathogenic infections. The development of AIDS in C499 confirmed that pathogenic HIV infection can occur in experimentally infected chimpanzees (29). In addition, we recently described progressive HIV infection in other chimpanzees within the Yerkes cohort (30). Transfusion of blood from C499 to an uninfected chimpanzee, C455, resulted in a dramatic decline in CD4⁺ T cells in the latter animal, which suggested the presence of a novel, chimpanzee-pathogenic HIV-1 isolate. However, because a blood transfusion was utilized, the role of other factors in CD4⁺ T-cell decline was unknown.

In this study we sought to confirm that the virus that evolved in C499 and was passed through C455 was consistently pathogenic for chimpanzees. To address this issue, an HIV-1 stock prepared from the plasma of C455 (26) was utilized for

the inoculation of two chimpanzees: one uninfected and one previously infected with HIV-1_{LAV}. The kinetics of HIV-1_{NC} infection in naive chimpanzee C534 were remarkably similar to those observed for the transfusion recipient C455. Each animal experienced a rapidly pathogenic course of infection, characterized by high plasma virus loads and rapid and sustained CD4⁺ T-cell loss, findings reminiscent of SHIV89.6p infection in rhesus macaques (31).

It is uncertain why we observed such great disparity between the plasma virus load (7.4 log₁₀ RNA copies/ml on the day of biopsy) and the cell-associated virus burden in the lymph node biopsy specimen collected from C534 at 3 weeks postinoculation. Variation in the level of virus replication among individual lymphoid organs may account for this discrepancy. Recent studies using the SIV-macaque model have shown that the intestinal tract is a major site of viral replication and CD4⁺ T-cell depletion at early stages of infection (35, 36). Alternatively, peripheral CD4⁺ T-cell depletion occurred very rapidly in C534 (101 CD4⁺ T cells/μl on the day of biopsy). The rate and degree of CD4⁺ T-cell loss combined with the emerging antiviral immune response at 3 weeks postinoculation may have depleted this node of target cells, resulting in low numbers of surviving HIV⁺ cells on the day of biopsy.

The slower rate of CD4⁺ T-cell loss and lower plasma virus burden observed for C459 are most likely the result of preexisting HIV-induced immune responses subsequent to HIV-1_{LAV} infection. Nevertheless, C459 experienced progressive CD4⁺ T-cell loss over the 96-week course of the study (1,200 cells/μl at inoculation, <200 cells/μl at 96 weeks). Taken together, the persistently elevated virus loads and rapid and sustained CD4⁺ T-cell loss observed following the inoculation of animals C455, C534, and C459 confirm that virus derived from C499 is more pathogenic for chimpanzees than previous HIV isolates.

The fact that CD4⁺ T-cell decline was induced in a chimpanzee in the face of an apparently apathogenic HIV-1 infection has implications for vaccine development. In essence, pathogenic HIV-1_{NC} infection was initiated in C459 despite "attenuated" immunization with HIV-1_{LAV}. Preexisting immunity and virus infection were unable to prevent the challenge with a pathogenic virus. Still, these results must be interpreted in light of the route and dose of virus administered to these chimpanzees.

Previous work by others suggested that virus derived from C499 is pathogenic for chimpanzees (6); however, the results presented here indicate that HIV-1_{JC/NC} expresses a more chimpanzee-pathogenic phenotype than does HIV-1_{JC499}. After 3 or more years of infection, however, none of the viruses derived from C499 have induced AIDS in chimpanzees, which may allay concerns over the use of such virulent isolates in chimpanzees (A. M. Prince, Letter, AIDS Res. Hum. Retroviruses **13**:1259, 1997; A. M. Prince, J. Allan, L. Andrus, B. Brotman, J. Eichberg, R. Fouts, J. Goodall, P. Marx, K. K. Murthy, S. McGreal, and C. Noon, Letter, Science **283**:1117-1118, 1999). The rapid CD4⁺ T-cell depletion and the high viral loads induced in animals infected with HIV-1_{NC} provide markers for evaluating vaccine efficacy in chimpanzees that were not previously available. Further studies are aimed at defining the mechanisms of HIV-1_{NC} pathogenesis and comparing cellular and humoral immune responses against HIV between these chimpanzees and nonprogressor chimpanzees in the Yerkes cohort.

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