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Changes in Human Immunodeficiency Virus Type 1 Envelope Glycoproteins Responsible for the Pathogenicity of a Multiply Passaged Simian-Human Immunodeficiency Virus (SHIV-HXBc2)

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In vivo passage of a poorly replicating, nonpathogenic simian-human immunodeficiency virus (SHIV-HXBc2) generated an efficiently replicating virus, KU-1, that caused rapid CD4$^+$ T-lymphocyte depletion and AIDS-like illness in monkeys (S. V. Joag, Z. Li, L. Foresman, E. B. Stephens, L.-J. Zhao, I. Adany, D. M. Pinson, H. M. McClure, and O. Narayan, J. Virol. 70:3189–3197, 1996). The *env* gene of the KU-1 virus was used to create a molecularly cloned virus, SHIV-HXBc2P 3.2, that differed from a nonpathogenic SHIV-HXBc2 virus in only 12 envelope glycoprotein residues. SHIV-HXBc2P 3.2 replicated efficiently and caused rapid and persistent CD4$^+$ T-lymphocyte depletion in inoculated rhesus macaques. Compared with the envelope glycoproteins of the parental SHIV-HXBc2, the SHIV-HXBc2P 3.2 envelope glycoproteins supported more efficient infection of rhesus monkey peripheral blood mononuclear cells. Both the parental SHIV-HXBc2 and the pathogenic SHIV-HXBc2P 3.2 used CXCR4 but none of the other seven transmembrane segment receptors tested as a second receptor. Compared with the parental virus, viruses with the SHIV-HXBc2P 3.2 envelope glycoproteins were more resistant to neutralization by soluble CD4 and antibodies. Thus, changes in the envelope glycoproteins account for the ability of the passaged virus to deplete CD4$^+$ T lymphocytes rapidly and specify increased replicative capacity and resistance to neutralization.

The human immunodeficiency viruses type 1 (HIV-1) and HIV-2 cause AIDS by depleting host CD4$^+$ T lymphocytes (2, 11, 17, 20). HIV-1 infection represents a major public health problem, afflicting an estimated 30 million people worldwide (according to UNAIDS and The World Health Organization). Efforts to understand HIV-induced disease and to develop an effective vaccine against HIV-1 will require animal models. The infection of Asian macaques by simian immunodeficiency viruses (SIV) can result in AIDS-like disease and therefore has been extremely useful for studies of the pathogenesis of primate immunodeficiency viruses (27). However, differences between the HIV-1 and SIV envelope glycoproteins limit the utility of the SIV-macaque model for studying envelope glycoprotein determinants of pathogenicity and for testing vaccine strategies directed against the viral glycoproteins.

To address these limitations, chimeric simian-human immunodeficiency viruses (SHIVs) containing the *tat*, *rev*, *vpu*, and *env* genes of HIV-1 have been constructed and shown to infect macaques (21, 28, 31). The efficiency of SHIV replication in macaques is greatly influenced by the sequence of the HIV-1 envelope glycoproteins, which have been shown to specify viral tropism and sensitivity to neutralizing antibodies (7–9, 26, 36, 41, 44–46, 48, 52, 55). These properties differ between HIV-1 viruses that are primary (for example, those that were passaged only in peripheral blood mononuclear cells [PBMC]) and viruses that were adapted to replicate in immortalized cell lines. The latter, laboratory-adapted viruses are typically more sensitive to neutralizing antibodies than are primary viruses (52). All HIV-1 isolates utilize CD4 as a receptor; primary viruses use the CCR5 chemokine receptor as a second receptor, while laboratory-adapted viruses typically use CXCR4 (1, 10, 13–15, 18).

SHIV chimerae constructed with the *env* gene from a laboratory-adapted HIV-1 isolate, HXBc2, replicated efficiently in rhesus monkey PBMC in culture. However, SHIV-HXBc2 viruses replicated poorly in rhesus monkeys, and no pathogenic consequences were observed up to 2 years after infection (28). Although SHIV constructs expressing some primary virus envelope glycoproteins replicated more efficiently in rhesus monkeys, these infections were also without pathogenic consequences (40).

Serial passage of nonpathogenic SHIVs in vivo has generated viruses that cause rapid depletion of CD4$^+$ T lymphocytes and AIDS-like illness in macaques (24, 40). SHIV (KU-1) was generated by serial bone marrow transfer from animals originally infected with the nonpathogenic SHIV-HXBc2. Infection with KU-1 resulted in dramatic CD4$^+$ T-lymphocyte depletion within 4 weeks and AIDS-like illness in 70% of infected pigtailed macaques (24, 49). The replication level of the KU-1 virus in infected macaques was significantly increased compared with that of the SHIV-HXBc2 virus. Analysis of the

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uncloned KU-1 virus revealed several changes in the *vpr*, *tat*, *rev*, *env*, and *nef* genes and in the long terminal repeats (LTRs) compared with the parental SHIV-HXBc2 virus (reference 50 and unpublished observations). In addition, the altered initiation codon in the *vpu* gene of the parental SHIV-HXBc2 virus was found to be restored in the KU-1 virus (50). A functional *vpu* gene is not sufficient for rendering the SHIV-HXBc2 virus pathogenic (29), suggesting that some combination of *vpr*, *tat*, *rev*, *env*, *nef*, and/or LTR changes might contribute to the pathogenicity of the KU-1 virus. Here we test the hypothesis that changes in the KU-1 envelope glycoproteins contribute to the pathogenicity of the virus, and we investigate the functional consequences of the observed *env* sequence changes.

MATERIALS AND METHODS

Viruses and cells. The KU-1 virus stock was obtained from Opendra Narayan through the National Institutes of Allergy and Infectious Diseases AIDS Research and Reference Reagent Program. $SIV_{mac}239$ and $SIV_{mac}316$ viruses were provided by Ronald Desrosiers at the New England Regional Primate Research Center.

HeLa, COS-1, HEK 293, and Cf2Th cells were obtained from the American Type Culture Collection.

Rhesus macaque PBMC were purified by Ficoll-Paque density gradient centrifugation of fresh rhesus macaque blood (obtained from the New England Regional Primate Research Center). Contaminating erythrocytes were lysed with ACK solution (150 mM NH_4Cl, 7 mM $NaHCO_3$; pH 7.2). Cells were washed three times with phosphate-buffered saline (PBS) and resuspended in RPMI 1640 complete medium plus 10% fetal calf serum, penicillin, and streptomycin. Cells were then stimulated for 2 days with 1 μg of phytohemagglutinin per ml and 10% interleukin-2 (Hemagen Diagnostics, Inc., Columbia, Md.) 24 h prior to infection.

Blood-derived rhesus macrophages were purified as previously described (49). Briefly, rhesus PBMC were grown at 37°C and 5% CO_2 in macrophage driving medium (MDM) (75% RPMI containing 10% human serum, 15% conditioned L929 medium, 55 μM β-mercaptoethanol, 10 mM HEPES, and 50 U of human granulocyte-macrophage colony stimulating factor [GM-CSF] and 4 U of macrophage colony-stimulating factor [M-CSF] per ml [Genzyme, Inc.]), replenishing with fresh MDM every third day for 2 weeks prior to infection.

Plasmids. The *env* fragment of the KU-1 virus was cloned as follows. Proviral DNA from KU-1-infected CEMx174 cells was purified by using the MicroTurboGen Genomic DNA Isolation Kit (Invitrogen, San Diego, Calif.) kit. A 3.64-kb PCR fragment containing unique *Asp*718 and *Not*I sites was generated using *Pfu* DNA polymerase and forward GBK1009 (5′-TTATATAAATAAGCGGCCGC GTACCGAGTTGACCAGGCG-3′) and reverse GBK1010 primers (5′-ATTG TGGGTCACAGTCTATTAT-3′). To obtain the consensus KU-1 sequence in the *env* region, the 3.64-kb fragment was purified from an agarose gel with the Gene Clean II kit (Bio 101, San Diego, Calif.) and sequenced by automated DNA sequencing at the Dana-Farber Cancer Institute Molecular Biology Core Facility. The following primers were used for DNA sequencing: GBK96-3 (5′-GTTTGGGCCACACATGCC-3′), 96-4.HXBc2 (5′-GGTGCAGAAAGAATA TGC-3′), GBK96-5 (5′-GCAGTCTAGCAGAAGAAG-3′), GBK96-6 (5′-CAA TCCTCAGGAGGGGAC-3′), GBK96-7.HXBc2 (5′-CGAGATCTTCAGACTT GG-3′), GBK96-8 (5′-GTTGCAACTCACAGTCTG-3′), GBK96-9 (5′-GGCA AGTTTGTGGAATTGG-3′), and GBK96-47.HXBC2 (5′-GCATATTCTTTCT GCACC-3′).

For cloning the KU-1 *env* gene, the purified 3.64-kb PCR fragment was digested with *Asp*718 and *Bam*HI. The resulting fragment was cloned into the pSVIIIenv expression vector (22). The inserts from three independent clones were sequenced. The 3.2 clone, which contained all of the consensus changes found in the KU-1 *env* sequence (50), was selected for this study.

The plasmids containing the 5′ and 3′ halves of the SHIV-HXBc2 provirus have been previously described (28). The Vpu^+ 3′ proviral clone (p3′u+SHIV) was used in these studies. The *Asp*718-*Bam*HI fragment from the KU-1 3.2 *env* clone in pSVIIIenv was subcloned into the p3′u+SHIV proviral plasmid to create the p3′SHIV-HXBc2P 3.2 plasmid.

The pHXBH10ΔenvCAT plasmid contains an *env*-defective, Vpu-expressing provirus with a chloramphenicol acetyltransferase (CAT) gene replacing the natural *nef* gene (53). The pSHIVΔenvCAT plasmid contains an *env*-defective SHIV provirus that expresses CAT. The pSHIVΔenvCAT plasmid was made by cloning an *env*-deleted, HIV-1 HXBc2 *Sal*I-*Bam*HI fragment containing *tat* and *rev* sequences into a plasmid containing an $SIV_{mac}239$ provirus. The CAT gene-containing *Bam*HI fragment was cloned into the *Bam*HI site, inactivating the *rev* gene.

Plasmids encoding human CD4 (pCD4) and the human chemokine receptors CCR5 and CXCR4 have been previously described (5, 10).

The pSVTat plasmid expressing the HIV-1 Tat was previously described (47) and was kindly provided by Heinrich Göttlinger.

Antibodies, SDF-1, and soluble CD4. Purified human soluble CD4 (sCD4) was donated by Raymond Sweet (SmithKline Beecham, King of Prussia, Pa.). Human stromal cell-derived factor 1 (SDF-1) was supplied by Timothy Springer and Ian Clarke-Lewis (3, 36). The IgG1b12 antibody, which recognizes the CD4 binding site of the HIV-1 gp120 envelope glycoprotein, was donated by Dennis Burton and Carlos Barbas III (6). The F105 antibody, which also recognizes the CD4 binding site of the HIV-1 gp120 envelope glycoprotein, was a kind gift from Marshall Posner (39). The AG1121 antibody, which recognizes the V3 loop of the HIV-1 gp120 envelope glycoprotein, was purchased from AGMED, Inc., Bedford, Mass. Human sera recognizing HIV-1 envelope glycoproteins were pooled from HIV-1-infected individuals.

Virus replication assays. To generate the Vpu^+ SHIV-HXBc2 and SHIV-HXBc2P 3.2 viruses, the p5′SHIV and either p3′u+SHIV or p3′SHIV-HXc2P 3.2 plasmids, respectively, were digested with *Sph*I and then ligated. The ligated DNA was transfected into CEMx174 cells, and viruses were harvested at the peak of viral replication.

Equivalent amounts of virus, measured in reverse transcriptase units, were used to infect rhesus PBMC and macrophages. To detect virus production, cell-free supernatants from infected rhesus PBMC were harvested and assayed for reverse transcriptase activity as previously described (28). An aliquot of the cell-free supernatants from SHIV-infected rhesus macrophage cultures was harvested daily and replaced with fresh MDM. GM-CSF and M-CSF were added every third day. The supernatants were then assayed for the presence of the SIV p27 core protein by utilizing an SIV p27 enzyme-linked immunoassay kit (Coulter Corp., Hialeah, Fla.).

***env* complementation assays.** To measure a single round of infection, recombinant viruses expressing CAT were produced by cotransfecting the pSVIIIenv plasmid expressing the envelope glycoproteins of interest with either the pSHIV-CAT plasmid (for monkey and canine target cells) or the pHXBH10ΔenvCAT plasmid (for other target cells). Recombinant viruses made with pSHIVΔenvCAT were generated in HEK 293 cells, while recombinant viruses made with pHXBH10ΔenvCAT were generated in either HeLa or COS-1 cells. Recombinant viruses were normalized for reverse transcriptase activity and used to infect target cells. The target cells were (i) Cf2Th canine thymocytes transfected with plasmids expressing CD4 and chemokine receptors, (ii) CEMx174 cells, or (iii) rhesus PBMC. The Cf2Th cells were cotransfected either with pCD4 and an empty pcDNA3.1 control or with pCD4 and a pcDNA3.1 plasmid expressing either human CXCR4 or CCR5 by the calcium phosphate method (12). CAT activity was measured in the target cells as previously described (22).

Animal inoculation. Three rhesus monkeys (*Macaca mulatta*) were inoculated intravenously with approximately 10^6 reverse transcriptase units of the SHIV-HXBc2P 3.2 virus generated in CEMx174 cells as described above. The rhesus monkeys were maintained in accordance with the guidelines of the Committee on Animals for the Harvard Medical School and the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health, 1985). The monkeys were anesthetized with ketamine-HCl for all blood sampling, biopsies, and inoculations.

Lymphocyte phenotyping. Peripheral blood lymphocytes obtained from the infected animals were phenotyped for CD4 (OKT4-FITC; Ortho Diagnostics Systems, Raritan, N.J.), CD8 (T8-phycoerythrin; Dako, Inc., Carpinteria, Calif.), and CD20 (B1-fluorescein isothiocyanate; Coulter Corp.), subsets by using a commercial whole-blood lysis kit (Coulter), as previously described (42). Absolute lymphocyte counts in blood were determined on an automated hematology analyzer (T540; Coulter) that provided a partial differential count.

Measurement of viremia. The concentration of SIV_{mac}p27 core antigen in heparinized plasma from the infected monkeys was determined by using a commercial kit (SIV Core [p27] Antigen EIA kit; Coulter).

Virus neutralization assay. Recombinant viruses containing either the HXBc2 or HXBc2P 3.2 envelope glycoproteins were generated as described above. Viruses were incubated with different concentrations of antibodies or sCD4 for 1 h at 37°C before the addition of CEMx174 target cells. Infected cells were harvested 3 days later, washed once in PBS, lysed in 0.25 M Tris-HCl (pH 7.5), and assayed for CAT activity as previously described (22).

SDF-1 inhibition assays were carried out using the same protocol, except that the rhesus PBMC target cells were incubated with 5 μg of SDF-1 per ml for 1 h at 37°C and 5% CO_2 prior to the addition of the recombinant viruses.

Radioimmunoprecipitation of envelope glycoproteins. Approximately 10^6 COS-1 cells were cotransfected with the pSVTat plasmid and the pSVIIIenv plasmid expressing either the HXBc2 or the HXBc2P 3.2 envelope glycoproteins. At 48 h after transfection, the cells were labeled with [^{35}S]cysteine for 12 h and then lysed in 0.5% Nonidet P-40, 0.5 M NaCl, and 10 mM Tris-HCl (pH 7.5). Cell lysates were used for immunoprecipitation by a mixture of sera from HIV-1 infected individuals or by monoclonal antibodies, as previously described (39, 54).

GenBank/EMBL accession number. The full proviral sequence of the SHIV-HXBc2P 3.2 clone has been submitted to GenBank under accession no. AF041850.

RESULTS

Amino acid changes in the KU-1 envelope glycoproteins. To examine the possible contribution of changes in the viral envelope glycoproteins to the evolution of a pathogenic SHIV, an *env* fragment was cloned from cells infected with the pathogenic SHIV isolate, KU-1 (24). The cloned *env* fragment encodes the gp120 exterior glycoprotein and the ectodomain and transmembrane segments of the gp41 glycoprotein. Figure 1 shows the predicted amino acid sequence encoded by the cloned *env* fragment and compares that sequence to the parental HXBc2 sequence and to the consensus sequence of the KU-1 virus. The cloned *env* fragment (clone 3.2) encodes envelope glycoproteins identical in sequence to that of the KU-1 consensus. With respect to the parental HXBc2 envelope glycoproteins, 12 amino acid differences were seen. Most of these changes were located in the variable regions (V1 to V5) of the gp120 glycoprotein. Interestingly, a new potential N-linked glycosylation site in the KU-1 V1 variable loop was created, while a glycosylation site at asparagine-276 was lost. Three amino acid changes in gp41, two of which are encoded by the cloned *env* fragment, were found. Both changes in the gp41 ectodomain alter potential N-linked glycosylation sites, although the more C terminal of these sites is near or within the membrane-spanning region of gp41 and therefore is not utilized.

Replication of SHIV-HXBc2P 3.2 in activated rhesus PBMC and macrophages. To study the functional consequences of changes in the envelope glycoproteins found in the KU-1 virus, Vpu^+ SHIV variants that differed only in *env* sequences were created (Fig. 1B). The replication of the parental SHIV-HXBc2 and SHIV-HXBc2P 3.2 viruses in rhesus monkey PBMC was examined. The uncloned KU-1 virus was also included in the experiment. As shown in Figure 2A, the KU-1 virus exhibited faster replication kinetics than did the molecularly cloned viruses. The SHIV-HXBc2P 3.2 virus replicated faster and achieved higher levels of virus production than did the SHIV-HXBc2 virus. Thus, changes in the envelope glycoproteins of the KU-1 virus are associated with faster replication kinetics in activated rhesus monkey PBMC.

Previous studies indicated that the KU-1 virus replicated more efficiently than the parental SHIV-HXBc2 in blood-derived monkey macrophages (50). To examine whether the envelope glycoproteins determined this phenotype, the replication of the KU-1 virus, SHIV-HXBc2, and SHIV-HXBc2P 3.2 was compared in rhesus monkey peripheral blood macrophages. $SIV_{mac}316$, which replicates efficiently in primary macrophages, and $SIV_{mac}239$, which does not replicate well in primary macrophages, were included as additional controls (34). Figure 2B shows that the KU-1 virus replicated as efficiently as the macrophage-tropic $SIV_{mac}316$ isolate, confirming previous observations (50). The SHIV-HXBc2 and SHIV-HXBc2P 3.2 viruses replicated comparably in primary macrophages and at lower levels than those attained by the KU-1 and $SIV_{mac}316$ viruses. The $SIV_{mac}239$ isolate, as expected, replicated very poorly in primary macrophages. These results indicate that the KU-1-associated changes in the envelope glycoproteins are not sufficient to account for the increased replicative efficiency of KU-1 in monkey macrophages.

The ability of SHIV-HXBc2P 3.2 to replicate in rhesus monkey PBMC more efficiently than the parental SHIV-HXBc2 virus could be attributed to either *cis* or *trans* effects of the *env* segment. To determine whether early events in the virus infection of PBMC were facilitated by the KU-1-associated changes in the envelope glycoproteins, we examined the ability of recombinant viruses containing either the HXBc2 or the HXBc2P 3.2 envelope glycoproteins to infect rhesus PBMC in a single round. At two different multiplicities of infection, viruses with the HXBc2P 3.2 envelope glycoproteins infected rhesus PBMC more efficiently than did viruses with the HXBc2 envelope glycoproteins (Fig. 2C). Thus, the increased ability of SHIV-HXBc2P 3.2 to infect rhesus PBMC compared with SHIV-HXBc2 can be attributed to the increased efficiency with which the HXBc2P 3.2 envelope glycoproteins mediate early events in the infection process.

Chemokine receptor use by the HXBc2 and HXBc2P 3.2 envelope glycoproteins. To date, the only second receptor known to be utilized by the HXBc2 envelope glycoproteins, which are derived from a laboratory-adapted virus, is CXCR4 (10, 18). Since a broadened range of chemokine receptor use might increase the efficiency of virus entry, we examined the ability of recombinant viruses containing the HXBc2 and HXBc2P 3.2 envelope glycoproteins to enter Cf2Th canine thymocytes expressing CD4 and a number of known second receptors for primate immunodeficiency viruses. Figure 3A shows that the HXBc2P 3.2 envelope glycoproteins utilize CXCR4 as a second receptor. By contrast, the entry of viruses with either the HXBc2 or the HXBc2P 3.2 envelope glycoproteins into cells expressing CD4 and CCR5 was inefficient. As expected, viruses with $SIV_{mac}239$ envelope glycoproteins entered cells expressing CD4 and CCR5 but not those expressing CD4 and CXCR4 (32). The ability of viruses with the HXBc2 and HXBc2P 3.2 envelope glycoproteins to infect Cf2Th cells expressing CD4 and other seven transmembrane segment receptors (CCR2b, CCR3, STRL33, gpr 15, and gpr 1) that have been shown to act as efficient second receptors for primate immunodeficiency viruses (10, 14, 16, 30) was also examined. None of these proteins allowed the entry of viruses with either the HXBc2 or the HXBc2P 3.2 envelope glycoproteins into CD4-expressing Cf2Th cells (data not shown). Thus, we did not find evidence of qualitative differences in chemokine receptor use between the HXBc2 and the HXBc2P 3.2 envelope glycoproteins.

To examine the use of the CXCR4 chemokine receptor in rhesus PBMC by viruses with the HXBc2 and HXBc2P 3.2 envelope glycoproteins, infection of these cells was carried out in the presence of SDF-1, a CXCR4 ligand (3, 37). SDF-1 dramatically inhibited the infection of rhesus PBMC by viruses with both envelope glycoproteins (Fig. 3B). However, infection of rhesus PBMC by viruses with the $SIV_{mac}239$ envelope glycoproteins, which use CCR5 but not CXCR4 as a coreceptor (32), was not inhibited (data not shown). These results are consistent with the studies described above and indicate that CXCR4 is the major coreceptor used for entry into rhesus PBMC by viruses with the HXBc2 and HXBc2P 3.2 envelope glycoproteins.

Infection of rhesus macaques with SHIV-HXBc2P 3.2. SHIV-HXBc2 replicates at only low levels in rhesus or pig-tailed macaques and does not cause $CD4^+$ T-lymphocyte depletion in infected animals (29, 49). However, the KU-1 virus replicates more efficiently and causes rapid $CD4^+$ T-lymphocyte depletion in pig-tailed and rhesus macaques (24). To investigate whether the KU-1-associated changes in *env* sequences contribute to these in vivo phenotypes, three rhesus macaques were inoculated intravenously with the molecularly cloned SHIV-HXBc2P 3.2 virus. The pattern of acute viremia in these animals was typical for SIV- or SHIV-infected monkeys, with a peak at approximately 14 days after inoculation (Fig. 4). The level of p27 antigenemia was approximately 50 to 100 times that previously seen (41) in four rhesus monkeys infected with the SHIV-HXBc2 virus (Fig. 4).

The $CD4^+$ T lymphocytes in all three SHIV-HXBc2P 3.2-

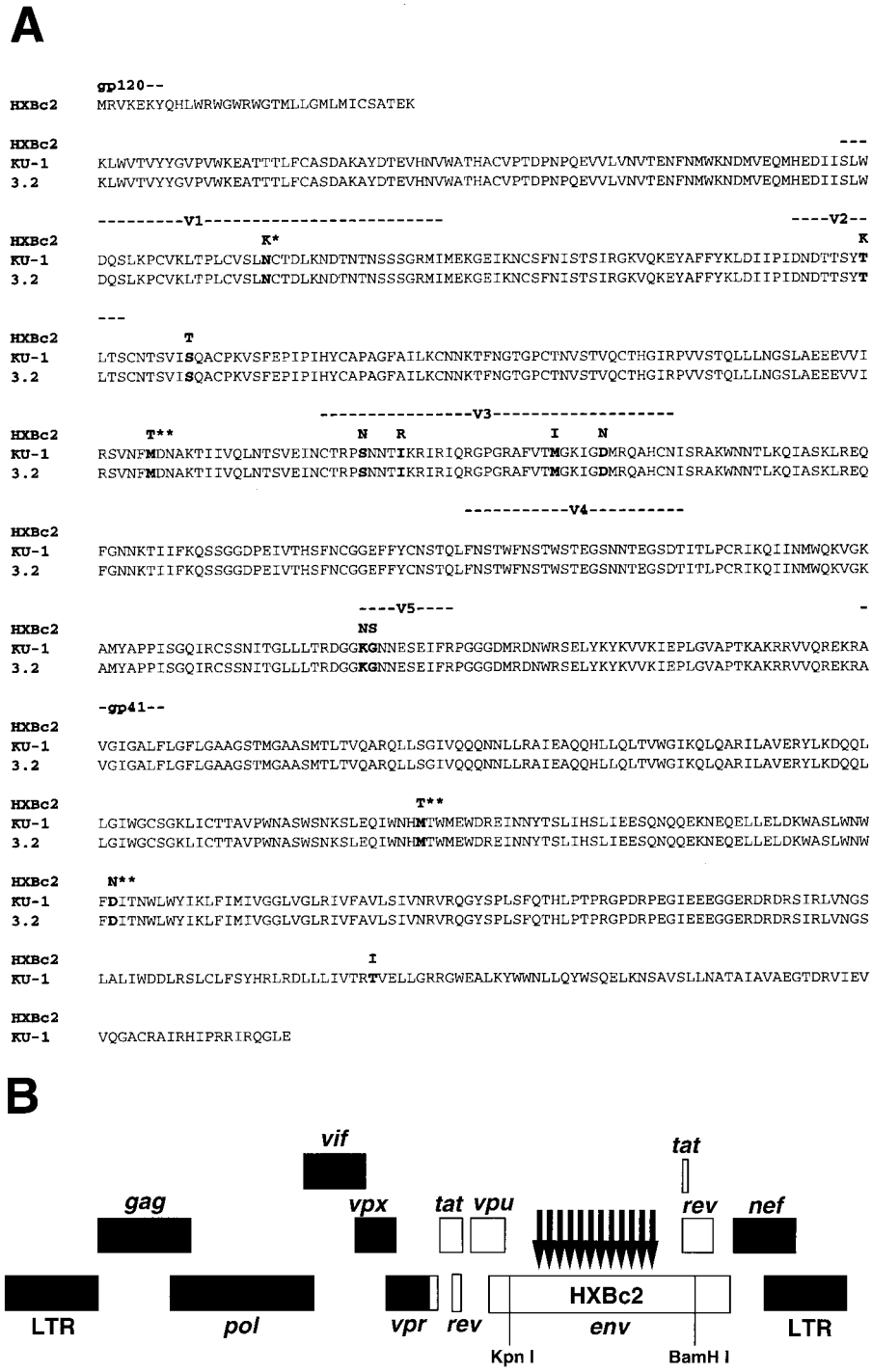

FIG. 1. Sequence of the KU-1 and SHIV-HXBc2P 3.2 viruses. (A) The predicted amino acid sequence of the envelope glycoproteins of the KU-1 virus, based on the consensus sequence of the *env* fragment amplified from KU-1-infected CEMx174 cells, is aligned with the HXBc2 sequence. The predicted sequence of the molecularly cloned HXBc2P 3.2 envelope glycoproteins is also shown. Residues in which differences are found between HXBc2 and the KU-1/HXBc2P 3.2 sequences are highlighted. The positions of the gp120 and gp41 boundaries are shown, as are the locations of the gp120 variable regions. Sequences in which the KU-1-associated changes result in the acquisition (*) or loss (**) of potential N-linked glycosylation are indicated. (B) The Vpu$^+$ SHIV-HXBc2 genome is shown, with the sequences derived from HIV-1 (HXBc2 strain) in white and those from $SIV_{mac}239$ in black. The positions of the 12 amino acid changes in the SHIV-HXBc2P 3.2 provirus are indicated by black arrows.

infected animals decreased dramatically, reaching nadirs of less than 45 cells per μl at day 17 after infection (Fig. 4). The CD4$^+$ T-lymphocyte counts in the three monkeys showed a slight recovery after the initial period of viremia but remained severely depressed. This pattern of CD4$^+$ T-lymphocyte depletion is typical of SHIV-infected animals that develop AIDS-like illness within 2 years of infection (24, 25, 40). In contrast, the four monkeys previously infected with the SHIV-HXBc2

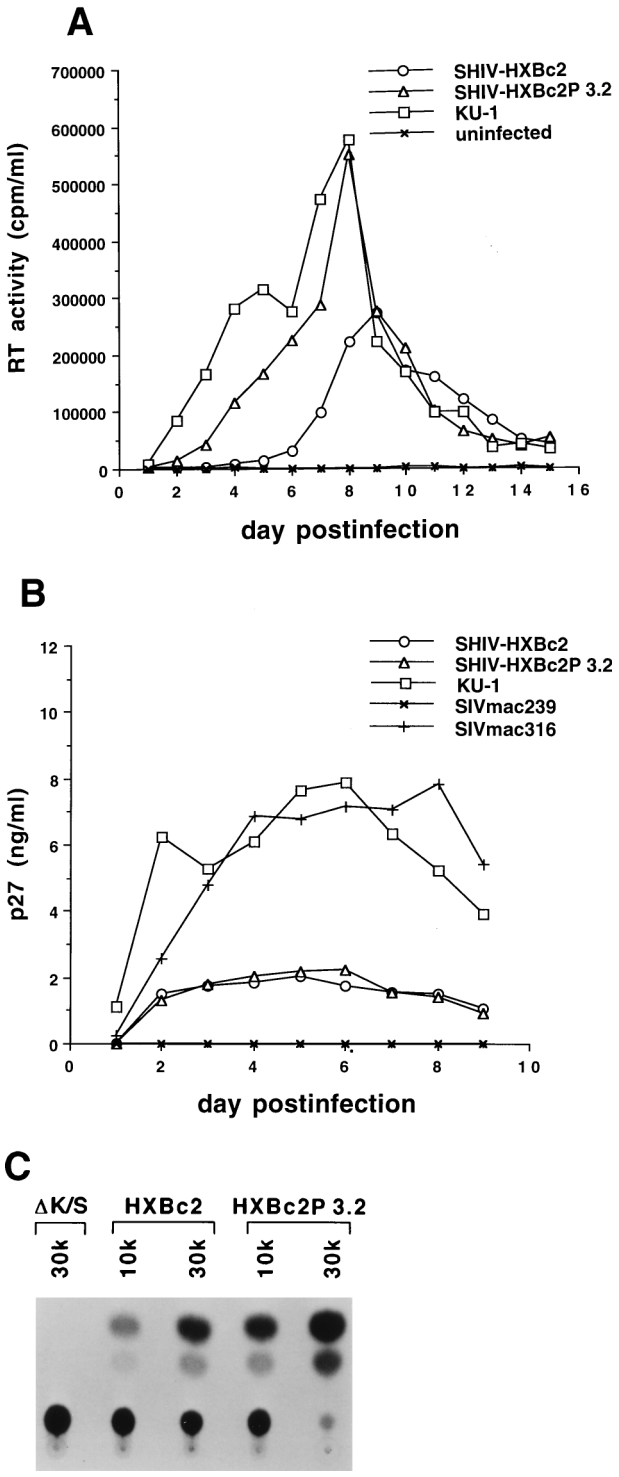

FIG. 2. Replication of viruses in activated rhesus monkey PBMC and blood-derived macrophages. (A) Activated rhesus monkey PBMC were either mock infected or infected with the uncloned KU-1 virus or the molecularly cloned SHIV-HXBc2 or SHIV-HXBc2P 3.2 viruses. Culture supernatants were harvested on the days indicated and assayed for reverse transcriptase activity. The results shown are representative of two independent experiments. (B) Blood-derived macrophages from a rhesus monkey were cultured for 2 weeks as described in Materials and Methods and then infected with the indicated SHIV and SIV viruses. Culture supernatants were harvested and assayed for p27 protein. Similar results were obtained in two independent experiments, one of which is shown here. (C) The CAT activity in the lysates of activated rhesus monkey PBMC infected with recombinant SHIV-CAT containing either no envelope glycoproteins (ΔK/S) or the HXBc2 or HXBc2P 3.2 envelope glycoproteins. Two different amounts of the latter viruses were used for infection. The amount (either 10,000 or 30,000 reverse transcriptase units) of each virus used for infection is indicated.

virus maintained normal levels of $CD4^+$ T lymphocytes (Fig. 4). Furthermore, two monkeys in the same colony inoculated at the same time as the three SHIV-HXBc2P 3.2-infected animals with another SHIV-HXBc2 variant exhibited no drop in $CD4^+$ T lymphocytes (data not shown).

Neutralization of viruses with the HXBc2 and HXBc2P 3.2 envelope glycoproteins. SHIV-infected monkeys generate neutralizing antibodies against HIV-1 (29). Because the laboratory-adapted HXBc2 parent virus is highly susceptible to neutralization by sCD4 and antibodies (52), some of the envelope glycoprotein changes associated with the pathogenic KU-1 virus might modulate neutralization sensitivity. To test this, recombinant viruses with the HXBc2 or HXBc2P 3.2 envelope glycoproteins were incubated with sCD4 and neutralizing antibodies prior to infection of CEMx174 lymphocytes. CEMx174 lymphocytes were chosen as target cells in this experiment because the basal level of infectivity of recombinant viruses with HXBc2 and HXBc2P 3.2 envelope glycoproteins did not significantly differ in these cells (Fig. 5A). As shown in Figure 5B, viruses with the HXBc2P 3.2 envelope glycoproteins were less sensitive to sCD4 than were viruses with the HXBc2 envelope glycoproteins. The sensitivity of these viruses to neutralization by monoclonal antibodies was examined. Two antibodies against the CD4 binding site, F105 and the unusually potent IgG1b12 antibody, were tested, as well as the AG1121 antibody against the gp120 third variable (V3) loop. Recombinant viruses containing the HXBc2 envelope glycoproteins were, as expected, highly susceptible to neutralization by all three antibodies (Fig. 5C to E). By contrast, viruses containing the HXBc2P 3.2 envelope glycoproteins were less efficiently neutralized by all three antibodies. The epitopes for all three antibodies were retained on the HXBc2P 3.2 envelope glycoproteins, since these antibodies precipitated the HXBc2P 3.2 envelope glycoproteins with an efficiency comparable to that seen for the HXBc2 envelope glycoproteins (Fig. 5F). Thus, the HXBc2P 3.2 envelope glycoproteins are less sensitive to neutralization than are the HXBc2 envelope glycoproteins. It is likely that this altered sensitivity is mediated by changes in the envelope glycoproteins outside of the gp120 epitopes for these antibodies.

DISCUSSION

The rapidity with which $CD4^+$ T lymphocytes are depleted in monkeys infected with pathogenic SHIV variants is remarkable. This model offers unique opportunities to understand the genetic elements that determine in vivo virus replication and $CD4^+$ T-lymphocyte depletion. Previous studies revealed multiple differences in LTR, *vpr*, *tat*, *rev*, *vpu*, *env*, and *nef* sequences between the parent SHIV-HXBc2 and the uncloned, pathogenic SHIV (KU-1) (49, 50). Here, using a molecularly cloned virus, we show that changes in the SHIV envelope glycoproteins are sufficient to confer on an otherwise nonpathogenic virus the ability to replicate efficiently in vivo and to cause rapid and persistent $CD4^+$ T-lymphocyte depletion. Twelve amino acid differences in the envelope glycoproteins distinguish the parent SHIV-HXBc2, which does not cause $CD4^+$ T-lymphocyte depletion in infected animals (24, 29), from SHIV-HXBc2P 3.2, which caused profound loss of $CD4^+$ T lymphocytes within 2 weeks of infection.

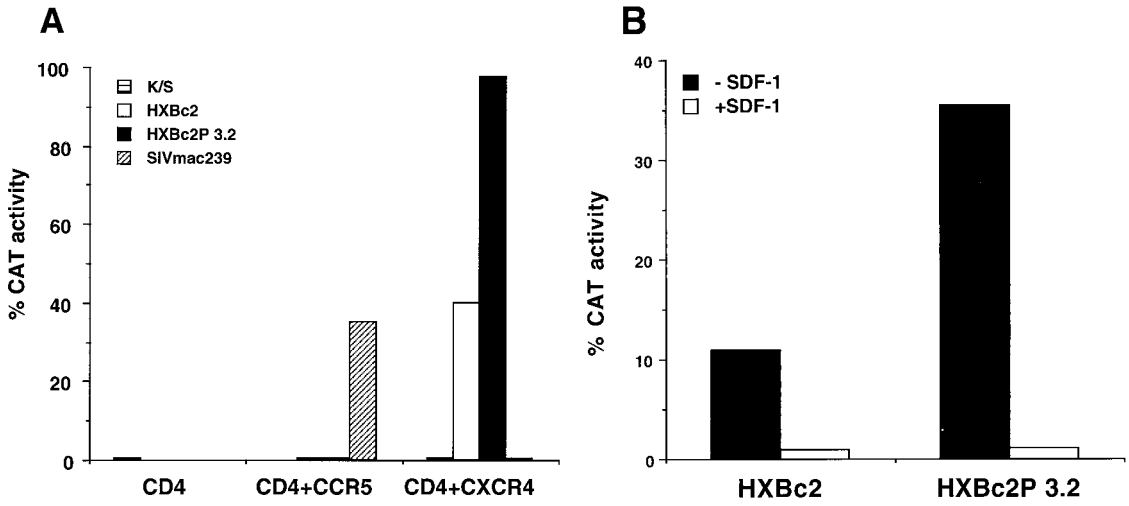

FIG. 3. Coreceptor use by the HXBc2 and HXBc2P 3.2 envelope glycoproteins. (A) The figure shows the CAT activity in the lysates of Cf2Th canine thymocytes expressing CD4 only, both CD4 and CCR5, or both CD4 and CXCR4 after incubation with recombinant SHIV-CAT containing the HXBc2, HXBc2P 3.2 or $SIV_{mac}239$ envelope glycoproteins. The percentage of chloramphenicol acetylated in each experiment is indicated. (B) The amount of CAT activity in the lysates of rhesus PBMC after incubation with viruses containing the HXBc2 or HXBc2P 3.2 envelope glycoproteins is shown. The target PBMC were incubated in the absence or presence of 5 μg of SDF-1 per ml. Similar results were obtained with PBMC from three different monkey donors.

The basis for the ability of the SHIV-HXBc2P 3.2 virus to cause acute $CD4^+$ T-lymphocyte depletion likely lies in the *env*-mediated increase in virus replication, which is manifest both in vivo and in tissue-cultured rhesus PBMC. Numerous studies in HIV-1-infected humans and SIV-infected monkeys have suggested that efficient virus replication is a prerequisite for $CD4^+$ T-lymphocyte destruction and pathogenicity. The poor in vivo replication of the parental SHIV-HXBc2 (28, 29, 41) limits the possibility of pathogenic consequences, at least within the 2-year period after infection. The 50- to 100-fold increase in acute viremia seen for the SHIV-HXBc2P 3.2 may allow the pathogenic process to impact a large percentage of the $CD4^+$ T-lymphocyte population. Further work will be required to understand the mechanistic basis of this replication increase. It does not, however, appear to result from a broadened use of chemokine receptors by the SHIV-HXBc2P 3.2 virus compared with the parental SHIV-HXBc2. Our results also indicate that the use of CXCR4 as a major coreceptor is sufficient to allow depletion of the vast majority of the $CD4^+$ T-lymphocyte population. This is consistent with the reported expression of CXCR4 on resting as well as activated T lymphocytes (4).

The uncloned pathogenic KU-1 virus replicated more efficiently than the SHIV-HXBC2P 3.2 virus in rhesus PMBC and macrophages, suggesting the possibility that genetic elements other than *env* contribute in a positive way to the replication of the KU-1 virus. Our results indicate, however, that such KU-1

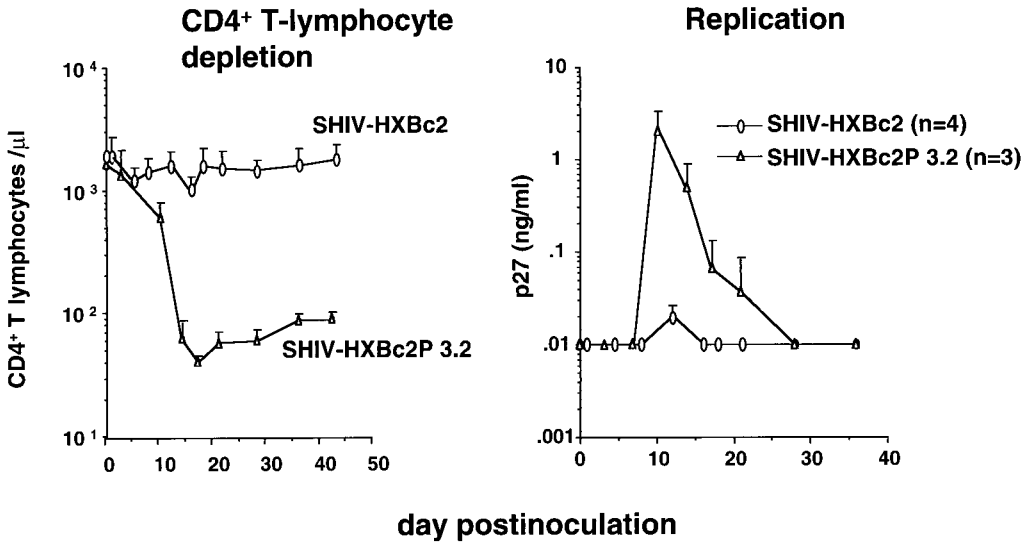

FIG. 4. Infection of rhesus macaques with SHIV-HXBc2P 3.2. The average amount of p27 antigen in the plasma of rhesus macaques inoculated intravenously with SHIV-HXBc2 and SHIV-HXBc2P 3.2 is shown for the first 5 weeks after infection. The average absolute number of $CD4^+$ T lymphocytes in the peripheral blood of SHIV-HXBc2- and SHIV-HXBc2P 3.2-infected macaques is shown. Results obtained in four SHIV-HXBc2-infected (previously shown in reference 41) and three SHIV-HXBc2P 3.2-infected monkeys are indicated. Error bars show the standard deviation of the combined animal experiments.

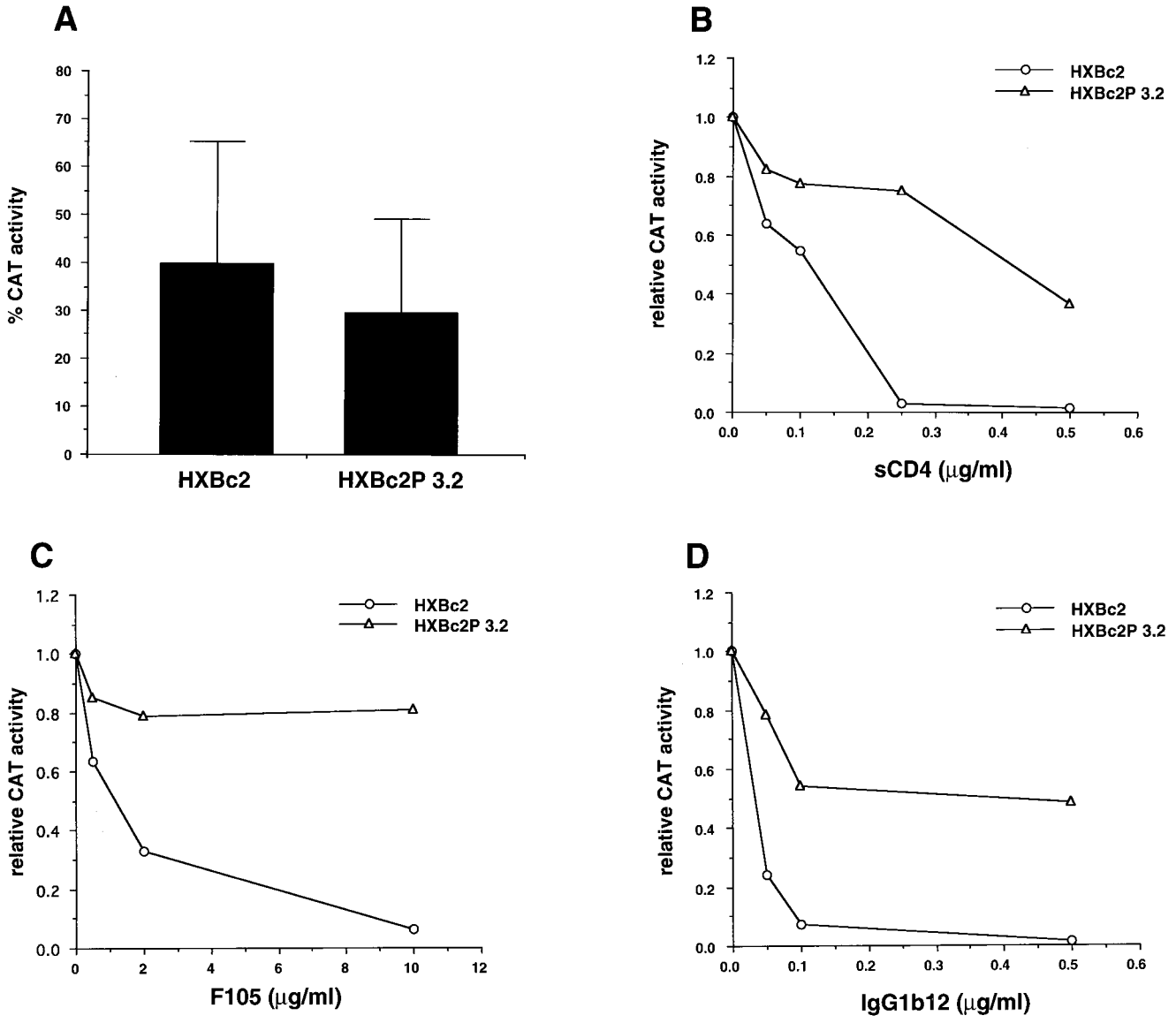

FIG. 5. Sensitivity of viruses with the HXBc2 and HXBc2P 3.2 envelope glycoproteins to neutralization by sCD4 and antibodies. In panel A, the level of infectivity of recombinant viruses containing either HXBc2 or HXBc2P 3.2 into CEMx174 cells is shown as the percentage of chloramphenicol conversion. Recombinant viruses containing either the HXBc2 or HXBc2P 3.2 envelope glycoproteins were incubated with sCD4 (B), the F105 antibody (C), the IgG1b12 antibody (D), or the AG1121 antibody (E) at the indicated concentrations prior to infection of CEMx174 cells. The results shown are representative of two independent experiments. In panel F, lysates of COS-1 cells expressing either the HXBc2 or HXBc2P 3.2 envelope glycoproteins were precipitated with pooled sera from HIV-1-infected individuals (PS) or with monoclonal antibodies. The 97.4 and 200-kDa protein molecular size markers (M) are shown. The gp160 and gp120 envelope glycoproteins are indicated.

elements and their associated phenotypes, including enhanced replication in primary macrophages, are not necessary for acute $CD4^+$ T-lymphocyte depletion in vivo.

In addition to mediating an increase in virus replication, the HXBc2P 3.2 envelope glycoproteins might contribute to the intrinsic ability of the virus to deplete $CD4^+$ T lymphocytes. A comparison of the plasma antigenemia and $CD4^+$ T-lymphocyte counts in rhesus macaques infected with SHIV-HXBc2P 3.2 or with SHIV-89.6 supports this possibility. Although SHIV-HXBc2P 3.2 did not achieve as high a level of viremia in one monkey as did SHIV-89.6 in rhesus monkeys (41), a precipitous decline in $CD4^+$ T-lymphocyte counts was seen only in the monkey infected with the former virus. Since the differences between SHIV-HXBc2P 3.2 and SHIV-89.6 reside within the *env* gene, this observation implies that the envelope glycoproteins may dictate the efficiency of $CD4^+$ T-lymphocyte destruction in this model. More carefully controlled in vivo studies in conjunction with in vitro characterization of the viral envelope glycoproteins may provide insights into the mechanistic basis of SHIV pathogenesis.

The envelope glycoproteins of the pathogenic SHIV-HXBc2P 3.2 render the virus less susceptible to neutralization by antibodies. In this respect, the laboratory-adapted HXBc2 envelope glycoproteins have evolved during in vivo passage to resemble the envelope glycoproteins of primary clinical isolates of HIV-1. It has been previously shown that monkeys infected with SHIV-HXBc2 initially generate strain-specific neutralizing antibodies that, with time, broaden to inhibit a

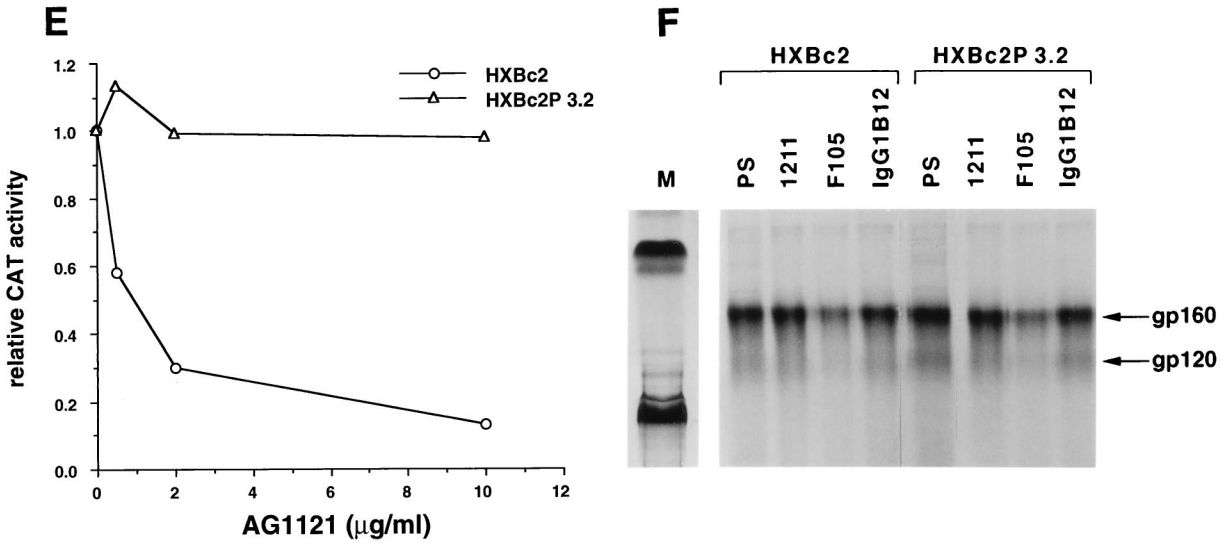

FIG. 5.—*Continued.*

variety of HIV-1 isolates (29). Indeed, some of the KU-1-associated changes in the envelope glycoproteins were previously seen in viruses isolated from SHIV-HXBc2-infected monkeys after several months of infection (29). While the advantage of neutralization resistance during acute infection is unclear, such resistance probably facilitates the establishment of persistent SHIV infections. Studies with antiviral therapy in HIV-1-infected humans and SIV-infected monkeys suggest that persistent virus replication is necessary for chronic $CD4^+$ T lymphopenia (23, 35, 38).

As has been documented for primary HIV-1 isolates (52), SHIV-HXBc2P 3.2 resistance to neutralizing antibodies is mediated by envelope glycoprotein changes outside of the antibody epitopes. The antibodies to the gp120 epitopes in the third variable loop or near the CD4 binding site recognized the HXBc2P 3.2 envelope glycoproteins. This result is consistent with the observation that most of the gp120 changes associated with conversion to a pathogenic virus (Fig. 1A) are outside of the regions thought to contribute to the epitopes for the antibodies examined (54). The gp120 variable loops, where most of the KU-1-associated changes occur, have been suggested to contribute to the relative resistance of some primary HIV-1 isolates to neutralization (51). This resistance is typically mediated by a decrease in the antibody accessibility of the epitopes in the context of the oligomeric envelope glycoprotein complex (19, 33, 43, 52).

The availability of a molecularly cloned pathogenic SHIV-HXBc2P 3.2 should assist future studies of viral determinants of in vivo replication and pathogenicity. The HXBc2P 3.2 envelope glycoproteins represent a defined antigen that should be useful in exploring the nature of protective immune responses against this pathogenic virus.

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