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An *env* Gene Derived from a Primary Human Immunodeficiency Virus Type 1 Isolate Confers High In Vivo Replicative Capacity to a Chimeric Simian/Human Immunodeficiency Virus in Rhesus Monkeys

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To explore the roles played by specific human immunodeficiency virus type 1 (HIV-1) genes in determining the in vivo replicative capacity of AIDS viruses, we have examined the replication kinetics and virus-specific immune responses in rhesus monkeys following infection with two chimeric simian/human immunodeficiency viruses (SHIVs). These viruses were composed of simian immunodeficiency virus SIVmac239 expressing HIV-1 *env* and the associated auxiliary HIV-1 genes *tat*, *vpu*, and *rev*. Virus replication was assessed during primary infection of rhesus monkeys by measuring plasma SIVmac p27 levels and by quantifying virus replication in lymph nodes using in situ hybridization. SHIV-HXBc2, which expresses the HIV-1 *env* of a T-cell-tropic, laboratory-adapted strain of HIV-1 (HXBc2), replicated well in rhesus monkey peripheral blood leukocytes (PBL) in vitro but replicated only to low levels when inoculated in rhesus monkeys. In contrast, SHIV-89.6 was constructed with the HIV-1 *env* gene of a T-cell- and macrophage-tropic clone of a patient isolate of HIV-1 (89.6). This virus replicated to a lower level in monkey PBL in vitro but replicated to a higher degree in monkeys during primary infection. Moreover, monkeys infected with SHIV-89.6 developed an inversion in the PBL CD4/CD8 ratio coincident with the clearance of primary viremia. The differences in the in vivo consequences of infection by these two SHIVs could not be explained by differences in the immune responses elicited by these viruses, since infected animals had comparable type-specific neutralizing antibody titers, proliferative responses to recombinant HIV-1 gp120, and virus-specific cytolytic effector T-cell responses. With the demonstration that a chimeric SHIV can replicate to high levels during primary infection in rhesus monkeys, this model can now be used to define genetic determinants of HIV-1 pathogenicity.

Emerging data suggest that virologic and immunologic events during the initial weeks following human immunodeficiency virus type 1 (HIV-1) infection may have long-term consequences on the course of disease progression. The burst of viral replication in the first days following HIV-1 infection is usually contained presumably by the early immune response (2, 4). However, a small subset of individuals never evidence significant control of virus spread and rapidly progress to clinical AIDS (1, 17). It is possible that long-term nonprogressors may control acute viral replication more effectively than those with a more rapid disease course. Thus, a better understanding of virus replication and immunologic responses during primary infection may be important for understanding HIV-1-induced disease.

Animal models will play a central role in facilitating the study of these early pathogenic events (9, 18). Although the simian immunodeficiency virus (SIV)/macaque monkey model has been a powerful system in which to study AIDS immunopathogenesis, its utility has been limited in addressing certain issues. The envelopes of HIV-1 and SIV are quite genetically divergent (23). Thus, SIV-infected macaques have not proven useful in identifying genetic determinants of HIV-1 Env responsible for pathogenicity. In addition, the HIV-1 and SIV envelope glycoproteins are antigenically distinct. Antibodies raised against either of these viral glycoproteins exhibit limited cross-reactivity with the envelope glycoprotein of the other virus (15, 28) and do not cross-neutralize (13). Consequently, it will be important to develop better animal models for studying AIDS pathogenesis.

Recombinant chimeric simian/human immunodeficiency viruses (SHIVs) hold promise for facilitating the study of such issues. Chimeric viruses consisting of SIVmac239 expressing HIV-1 genes have been shown to infect macaque monkeys (12, 21, 22). However, with only one exception (22), the recombinant viruses assessed to date in nonhuman primates have replicated to a much lower level than SIVmac. Monkeys infected with these viruses for more than 2 years have not shown any pathologic consequences (12, 20). In an attempt to create a SHIV that replicates more efficiently in vivo, we have gener-

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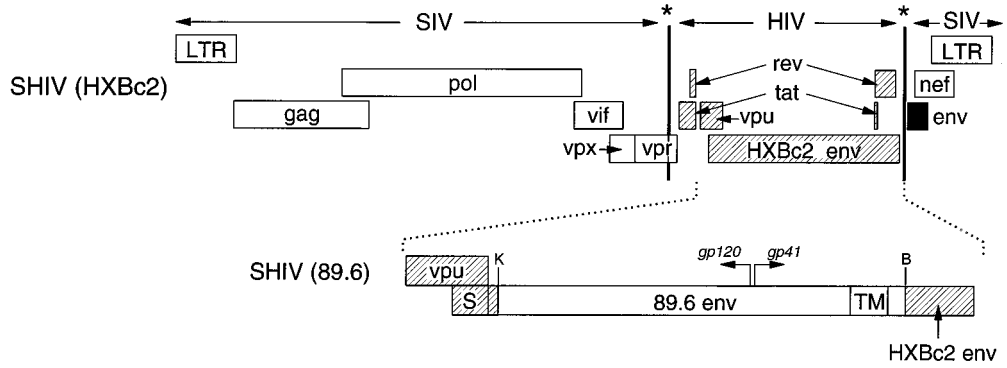

FIG. 1. Structures of the SHIV variants used in this study. The SHIV-HXBc2 chimera, which has an intact *vpu* gene (21), is shown at the top. The junctions of HIV-1 and SIVmac sequences are denoted by asterisks. The residual 3′ end of the SIV *env* gene is shown in solid black. LTR, long terminal repeat. The structure of the SHIV-89.6 chimera is shown at the bottom. Shaded sequences were derived from the HXBc2 isolate, and unshaded sequences were derived from the 89.6 isolate. The *Kpn*I (K) and *Bam*HI (B) restriction sites used for insertion of the 89.6 sequences into the SHIV-HXBc2 construct are shown. The signal peptides (S) and transmembrane region (TM) of the envelope glycoproteins are shown.

ated a recombinant virus by using SIVmac239 and the *env* gene of a cytopathic primary patient isolate of HIV-1, 89.6. In this present study, we have compared the virologic and immunologic consequences of primary infection with this SHIV-89.6 chimera and a SHIV constructed with an HIV-1 *env* gene derived from a T-cell line-passaged HIV-1 isolate, HXBc2.

MATERIALS AND METHODS

Plasmid construction. The structures of the chimeric viruses used in these studies are shown in Fig. 1. The p3′u+SHIV plasmid containing the HIV-1 HXBc2 *env* gene was used as the parent vector for the SHIV-89.6 construct. The fragment containing the *env* and *rev* gene segments of the HXBc2 virus from the *Kpn*I (5925) site to the *Bam*HI (8053) site was removed from the p3′u+SHIV plasmid. A *Bam*HI site was introduced into the 89.6 molecular clone (3) by PCR, and the 89.6 *Kpn*I-*Bam*HI *env*/*rev* fragment was used to replace the equivalent fragment deleted from the p3′u+SHIV plasmid. The resultant construct encodes an envelope glycoprotein with a chimeric gp41 intracytoplasmic tail and a chimeric Rev protein, due to the 89.6-HXBc2 junction at the *Bam*HI site. Both chimeric molecules have been previously demonstrated to be functional (37).

In vitro propagation and production of virus stocks. Chimeric viruses designated SHIV-HXBc2 and SHIV-89.6 were generated by ligating digested p5′SHIV with p3′u+SHIV and p3′89.6u+SHIV, respectively, and transfecting the ligation reaction into CEMx174 cells as described previously (21). The SIVmac239 (Nef-open) virus was generated as described previously (21). Chimeric viruses were propagated in rhesus monkey peripheral blood leukocytes (PBL) to compare the in vitro replication levels of the two different SHIVs and to create virus stocks for use in animal inoculations. Rhesus monkey PBL were isolated from heparinized blood and propagated in RPMI 1640 supplemented in 10% fetal bovine serum and 20 U of recombinant human interleukin-2 (Collaborative Research, Inc., Bedford, Mass.) per ml. Equivalent reverse transcriptase units of CEMx174-grown virus were used to initiate infection of rhesus monkey PBL. Virus replication in cultures was monitored every 3 to 4 days by reverse transcriptase assays as described previously (34). After removal of supernatants for reverse transcriptase assays, cells were suspended in sufficient fresh medium to maintain cell density between 10^5 and 10^6 cells per ml. Supernatants containing the highest levels of reverse transcriptase activity were pooled, and titers were determined on CEMx174 cells as previously described (21). This material was stored in liquid nitrogen and served as the stock virus for animal inoculations.

The in vitro growth of these chimeric viruses was also assessed in macrophages. Cultures of adherent mononuclear cells were established from bone marrow aspirates taken from normal rhesus monkeys as previously described (40). Cell cultures containing predominantly adherent mononuclear cells were inoculated with equivalent quantities of SHIV-89.6, SHIV-HXBc2, or the macrophage-tropic SIVmac 239/316 env (27), as determined by measuring p27 levels in virus stocks. Tissue culture supernatants were collected weekly and assayed for SIVmac p27 as a measure of virus replication.

Inoculation of rhesus monkeys with SHIV. The rhesus monkeys (*Macaca mulatta*) used in this study were maintained in accordance with the guidelines of the Committee on Animals for the Harvard Medical School and *Guide for the Care and Use of Laboratory Animals* (29). Animals were infected by intravenous inoculation with 400 50% tissue culture infective doses of either SHIV-HXBc2 or SHIV-89.6 that was propagated as described above. Monkeys were anesthetized with ketamine-HCl for all blood sampling and biopsies.

SIVmac p27 assay. The concentration of SIVmac p27 core antigen in heparinized plasma from animals after SHIV inoculation or in tissue culture supernatant was determined by using a commercial kit (SIV Core [p27] Antigen EIA kit; Coulter Corporation, Hialeah, Fla.).

Detection of viral DNA by PCR. Viral DNA was detected in peripheral blood mononuclear cells by using PCR amplification as described previously (31). Primers from the region of the SIVmac viral genome where the *pol* and *gag* genes overlap were used to produce a 200-bp product.

In situ hybridization for SHIV RNA in lymph nodes. In situ hybridization of lymph nodes was performed with a ^{35}S-labeled single-stranded (antisense) RNA probe of SIVmac239 (Lofstrand Laboratories, Gaithersburg, Md.). The clone was obtained in collaboration with Suzanne Gartner (Jackson Laboratories, Rockville, Md.) through the NIH AIDS Research and Reference Reagent Program. The probe was composed of fragments 1.4 to 2.7 kb in size and collectively represented approximately 90% of the SIV genome.

Lymph node biopsies obtained prior to and at weekly intervals after virus inoculation were fixed for 4 h in 4% neutral buffered formalin followed by 70% ethanol and then routinely dehydrated and embedded in paraffin without further exposure to formalin. Five-micrometer-thick sections were placed on slides coated with 3-aminopropylethoxysilane. Five sections from each lymph node were hybridized as previously described (31), with some modification. Briefly, the sections were dewaxed in xylene, rehydrated in 96 to 50% ethanol, and washed in diethylpyrocarbonate-treated water. Treatments with proteinase K and acetic anhydride were omitted. Sections were placed in prehybridization mixture (50% formamide, 0.5 M NaCl, 10 mM Tris HCl, [pH 7.4], 1 mM EDTA, 0.02% Ficoll-polyvinyl pyrrolidone-bovine serum albumin, 2 mg of tRNA per ml) for 2 h at 45°C. The prehybridization solution was replaced by hybridization cocktail (prehybridization mixture, 10% dextran sulfate, 2×10^6 dpm of radiolabeled probe per ml; boiled for 60 s and then chilled). Each section was layered with 4 μl/cm^2, covered with a coverslip, sealed with rubber cement, and kept at 45°C overnight. Sections were then washed sequentially with three changes each in 50% formamide–2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate), then in 2× SSC–0.01% Triton, and finally in 0.01× SSC. Sections were then digested with RNase (Boehringer GmbH, Mannheim, Germany) at 37°C for 40 min, washed again in 2× SSC, and dehydrated in 0.3 M ammonium acetate–70 to 96% ethanol. Slides were dipped in Kodak NTB-2 emulsion, exposed for 10 days at 4°C, developed in Kodak D-19 developer, counterstained with hemalaun, and mounted.

As a positive control, cytospin preparations of peripheral blood mononuclear cells infected with SIVmac were hybridized with the same probe. As a negative control, one section per lymph node for each time point was hybridized with a ^{35}S-labeled sense-strand probe. The sections were examined with an Axiophot Zeiss microscope equipped with epiluminescent illumination. Cells with at least 20 silver grains, which corresponded to a sixfold increase in silver grains over the background level, were scored as viral RNA positive. By using epiluminescent illumination, viral RNA-positive cells per section were counted with a 20× objective.

Immunophenotyping of rhesus monkey PBL. Monkey PBL were immunophenotyped flow cytometrically by a two-color, whole blood lysis technique using human leukocyte-specific monoclonal antibodies to recognize monkey CD4, CD8, and CD20 as previously described (33). For T-cell subsets, the monkey CD3-specific monoclonal antibody FN18 (30) was always used as the second fluorochrome. Absolute cell number was calculated from the lymphocyte count determined by an automated hematology analyzer (T540; Coulter Corporation). Preinoculation values for each lymphocyte subset of each monkey were determined by taking the mean of three independent measurements obtained during the week prior to inoculation. Pre- and postinoculation pairwise comparisons of

TABLE 1. Biological characterization of HIV-1 *env* donor viruses

Clone	Property (reference)			
	Origin	Tropism	Neutralization by monoclonal antibodies	Neutralization by recombinant soluble CD4
HXBc2	Laboratory-adapted strain (6, 35)	Primary T cells and many T-cell lines (35)	Sensitive (5, 37)	Sensitive (7, 37)
89.6	Primary patient isolate from peripheral blood mononuclear cells (3)	Primary T cells and macrophages, some T-cell lines (3, 16)	Resistant (37)	Resistant (37)

CD4/CD8 ratios were made by using Dunnett's test and determined to be significantly different when P was <0.05.

SHIV-specific antibody responses. HIV-1 envelope-specific antibodies induced by SHIV infection were quantitated by enzyme-linked immunosorbent assay (ELISA) with HIV-1 IIIB recombinant gp160 (rgp160; MicroGeneSys, Inc., Meriden, Conn.) as the antigen. Immunlon-2 plates were coated with rgp160 (1 μg/ml) in carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$ [pH 9.8]) overnight at 4°C. Solutions were aspirated, and the wells were filled with 100 μl of blocking buffer (Filter Paper Diluent; DuPont, Wilmington, Del.) containing 2.5% fetal bovine serum and incubated at 37°C for 4 h. Plates were washed four times with phosphate-buffered saline containing 0.05% Tween 20. Plasma samples were evaluated in duplicate at 1:50 dilution in wells containing borate buffer (0.1 M boric acid, 47 mM sodium borate, 75 mM NaCl, 0.05% [vol/vol] Tween 20) plus 2.5% fetal bovine serum. Color development was accomplished by using alkaline phosphatase-conjugated goat anti-monkey immunoglobulin G (Sigma Chemical Company, St. Louis, Mo.) followed by incubation with *p*-nitrophenylphosphate disodium hexahydrate (Sigma 104 phosphatase substrate) in diethanolamine buffer (0.9 M diethanolamine–7 mM $MgCl_2$ [pH 9.8] with concentrated HCl). Absorbance was measured at 405 nm.

Antibodies that neutralized SHIV-HXBc2 and HIV-1 MN were measured in MT-2 cells by using a cell killing assay as described previously (25). Virus stocks were prepared in H9 cells and titrated by p24 concentration and 50% tissue culture infective dose assay in MT-2 cells as described previously (25). A similar assay was used to measure neutralizing antibodies against SHIV-89.6 except that the stock titer was determined and assays were performed in CEMx174 cells. Serum samples were heat inactivated for 1 h at 56°C prior to assay.

In vitro T-lymphocyte proliferative response to rgp120. Ficoll-isolated PBL were cultured in RPMI 1640 containing 10% fetal calf serum and antibiotics at 10^5 cells per well in triplicate in 96-well round-bottom plates either with or without HIV-1 rgp120 (Intercel, Cambridge, Mass.) at a final concentration of 1 μg/ml in 0.2 ml. Cells were cultured for 6 days and pulse-labeled overnight with [^{3}H]thymidine. Cultures were harvested onto glass fiber filters by using an automated harvester (Tomtec, Orange, Conn.), and radiolabeled thymidine incorporation was determined by liquid scintillography measured with a Microbeta scintillation counter (Wallac, Gaithersburg, Md.).

SHIV-specific cytotoxic effector cell responses. Virus-specific cytolytic effector cell activity was determined as described previously (39). Briefly, PBL were isolated from heparinized blood or from lymph node biopsies and cultured for 3 days in RPMI 1640–10% fetal bovine serum supplemented with 5 μg of concanavalin A per ml and then expanded for an additional 3 days in RPMI 1640–10% fetal bovine serum supplemented with recombinant human interleukin-2. These PBL were used for standard ^{51}Cr release assays using autologous B-lymphoblastoid target cells infected with recombinant vaccinia viruses expressing either the HIV-1 *env* or SIVmac *gag* or *pol* gene products. Specific lysis was calculated by comparison with ^{51}Cr release of wild-type vaccinia virus-infected target cells.

RESULTS

In vitro replication of SHIV-HXBc2 and SHIV-89.6 in rhesus monkey PBL and macrophages. The cloned HIV-1 isolates that served as *env* donors (HXBc2 and 89.6) to create the two chimeric viruses have approximately 90% nucleotide and amino acid similarity within *env* (3). However, these HIV-1 isolates differed significantly in their in vitro biological activities (Table 1). The laboratory-adapted strain HXBc2 is tropic for both primary T cells and transformed T-cell lines and is sensitive to neutralization by Env-specific monoclonal antibodies and recombinant soluble CD4. The 89.6 virus represents a peripheral blood mononuclear cell-derived patient isolate of HIV-1 that was only briefly passaged on human lymphocytes (3). In contrast to HXBc2, 89.6 replicates in both macrophage and primary T-cell cultures. Unlike some primary HIV-1 isolates, the 89.6 virus replicates in some transformed T-cell lines. This virus was relatively resistant to neutralization by monoclonal antibodies and recombinant soluble CD4 (37). Finally, 89.6 was markedly more cytopathic to cultured cells than HXBc2 (3).

To assess the ability of rhesus monkey PBL to support replication of these chimeric SHIVs containing biologically divergent HIV *env* genes, lectin-activated lymphocytes were inoculated with equivalent reverse transcriptase units of SHIV-HXBc2, SHIV-89.6, or SIVmac239 (Fig. 2). While rhesus monkey PBL supported the replication of both chimeric viruses, SHIV-89.6 replication was significantly delayed compared with that of SHIV-HXBc2. The in vitro replication kinetics of SHIV-HXBc2 were only slightly delayed compared with those of the parent virus, SIVmac239. These replication

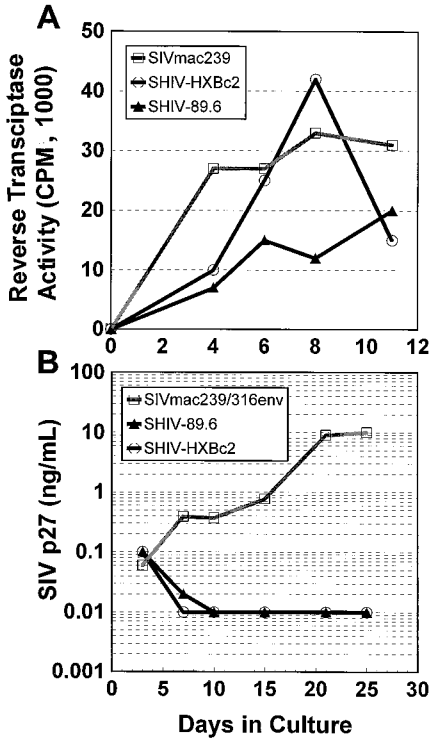

FIG. 2. Both SHIV-HXBc2 and SHIV-89.6 replicate in vitro in rhesus monkey PBL but not in rhesus monkey macrophages. (A) Lectin-activated rhesus monkey PBL were inoculated with SHIV-HXBc2 and SHIV-89.6, and virus replication was compared with that after inoculation with the parent virus SIVmac239. Virus replication was quantified by measuring reverse transcriptase activity in culture supernatants. (B) Bone marrow-derived macrophage cultures were established and inoculated with SHIV-HXBc2, SHIV-89.6, or the macrophage-tropic variant SIVmac239/316 env. Virus replication was assessed by measuring SIV p27 levels in the supernatant.

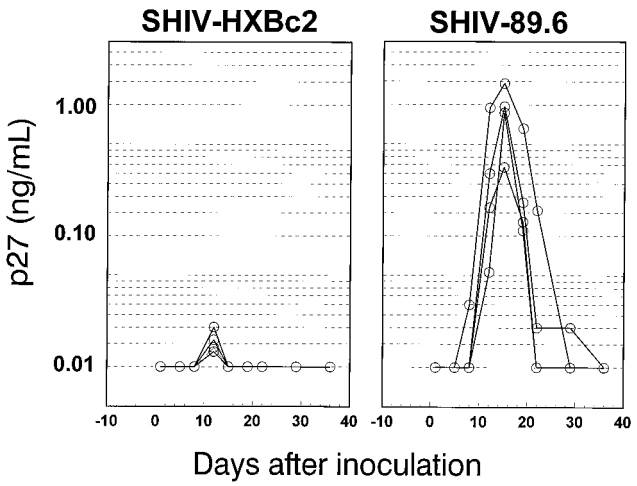

FIG. 3. Peak virus replication in vivo was 30- to 100-fold higher in monkeys inoculated with SHIV-89.6 than in those inoculated with SHIV-HXBc2. In vivo replication of SHIV was measured in eight monkeys by quantifying plasma SIVmac p27, using a commercially available ELISA.

patterns were reproduced in more than three independent experiments.

Measurable replication of SHIV-HXBc2 or SHIV-89.6 was not detected in bone marrow-derived macrophage cultures. These cultures did, however, support the growth of the macrophage-tropic variant SIVmac239/316 env.

Plasma SIVmac p27 levels in infected monkeys. To assess the replication of these two divergent SHIVs in vivo, two groups of four rhesus monkeys were inoculated intravenously with 400 50% tissue culture infective doses of either chimeric virus. Virus replication was determined in all animals by measuring SIVmac p27 in plasma samples collected every 3 to 4 days. In the four monkeys inoculated with SHIV-HXBc2, plasma p27 was measurable at only a single time point, 11 days after inoculation (Fig. 3). The viral antigen level in the plasma was approximately 0.01 to 0.02 ng/ml, just within the limits of detection of the assay. In contrast, viral antigen was repeatedly detected for 10 to 20 days postinoculation in the four monkeys inoculated with SHIV-89.6 (Fig. 3). Peak plasma p27 levels in these monkeys were 0.34 to 1.45 ng/ml, 30- to 100-fold higher than in the SHIV-HXBc2-infected animals.

Plasma p27 was not detected during the 30 to 70 days postinoculation in the animals in either group. To determine whether infection was persistent, DNA extracted from PBL was assessed for the presence of provirus by PCR amplification. Positive signals that we obtained in PBL specimens collected through day 70 in all monkeys inoculated with SHIV-HXBc2 and SHIV-89.6 indicated that infection was persistent in animals inoculated with both viruses (data not shown).

Histology and in situ hybridization studies of lymph nodes. Since lymph nodes are an important location of virus replication during primary SIV and HIV infection and a significant virus reservoir in chronic infection, we assessed the histologic changes and virus replication in lymph nodes during primary infection with these two SHIVs. A peripheral lymph node was excised prior to inoculation and at weekly intervals three times after inoculation in all animals.

The histologic appearance of lymph nodes obtained before inoculation and at 7 days after inoculation did not differ between the two groups of monkeys. In most animals, the B-cell zones contained mainly primary follicles, with only a few small hypocellular germinal centers surrounded by mantle zones. The paracortical zones were well developed in all animals and were made up predominantly of small lymphocytes. At 14 and 21 days postinoculation, histologic changes were evident in the lymph nodes. Interestingly, these changes were again similar in animals inoculated with SHIV-89.6 and SHIV-HXBc2. The lymph nodes from all animals demonstrated well-developed, regularly shaped germinal centers with zonation and were surrounded by a regular mantle zone (data not shown).

Virus replication in lymph nodes was also assessed in each group of monkeys by in situ hybridization for SIVmac RNA (Fig. 4 and 5). Hybridization signals were absent in biopsy specimens obtained prior to virus inoculation. At 7 days postinoculation, lymph nodes of only one of four animals inoculated with SHIV-HXBc2 showed cells with positive hybridization signals; lymph nodes of all four animals inoculated with SHIV-89.6 showed viral RNA-positive cells at this time. The number of productively infected cells was low, and distributions of infected cells in extrafollicular parenchyma were similar in the two animal groups.

At 14 days after inoculation, lymph nodes of three of the four animals that received SHIV-HXBc2 showed productively infected cells. However, the number of positive cells and the intensity of the hybridization signals were low (Fig. 4A). The one animal in this group with productively infected cells at day 7 remained positive but showed no increase in positive cell number or hybridization signal intensity. In contrast, lymph nodes of all four animals that received SHIV-89.6 demonstrated dramatic increases in the number of productively infected cells. Although the magnitude of this increase varied between SHIV-89.6-infected monkeys, all were characterized by high numbers of positive cells and high silver grain counts (Fig. 4B and 5). The majority of infected cells were found in the extrafollicular parenchyma and the sinuses, but productively infected cells were also present in germinal centers. Diffuse labeling of the germinal centers that exceeded the background level was not detectable at this time.

At 21 days postinoculation, the lymph nodes of animals inoculated with SHIV-HXBc2 continued to demonstrate low numbers of productively infected cells in the extrafollicular parenchyma, with no infected cells in germinal centers. In monkeys receiving SHIV-89.6, the number of productively infected cells had decreased markedly (Fig. 4C and 5). Infected cells were now seen not only in the extrafollicular parenchyma and sinuses but also in the germinal centers. In addition, diffusely distributed hybridization signals were present in the light zone of the germinal centers (Fig. 4C and D). This finding coincided with the appearance of envelope-specific antibodies and is consistent with follicular dendritic cell trapping of virus.

SHIV-specific antibody responses. Animals infected with SHIV-HXBc2 and SHIV-89.6 showed strikingly similar time courses for the appearance of envelope-specific antibodies identified by $gp160_{IIIB}$ ELISA; antibodies first became detectable 14 to 18 days postinoculation and peaked between days 29 and 36 for all animals in both groups (data not shown). Peak ELISA reactivity was stronger for plasma from animals infected with SHIV-HXBc2. The higher peak titers might have been observed in SHIV-HXBc2-infected animals because HXBc2 was derived from the IIIB strain of HIV-1. Thus, the antigen used in ELISA had greater sequence homology to SHIV-HXBc2 than to SHIV-89.6.

Neutralizing antibodies titers against SHIV-HXBc2, SHIV-89.6, and HIV-1 MN were assessed in all animals, using serum obtained 147 and 225 days postinoculation. The heparin used as the anticoagulant for plasma samples obtained prior to these time points was found to have potent antiviral activity that interfered with the measurement of neutralizing antibodies. As

FIG. 4. Magnitude of virus replication in lymph nodes correlated with plasma p27 levels, determined by in situ hybridization of lymph node specimens. (A) At 14 days after inoculation with SHIV-HXBc2, there are few productively infected cells (arrows). (B) At 14 days after inoculation with SHIV-89.6, many productively infected cells are apparent. Virus-producing cells are already present at the edge of a small germinal center (arrows); diffuse hybridization signals are absent. (C) At 21 days after inoculation with SHIV-89.6, there are fewer productively infected cells than at the earlier time point, and a diffuse hybridization signal is seen over the light zone of the germinal centers (arrows). (D) Higher magnification of a germinal center (asterisk) showing both diffuse hybridization signals and productively infected cells (arrows) 21 days after inoculation with SHIV-89.6. (A to C) Epipolarized light; viral RNA-positive cells appear as light areas; magnification, ca. ×82. (D) Combined epipolarized light and transillumination; positive hybridization signals are blue-green in epipolarized light; magnification, ca. ×218.

a result, plasma samples could not be used in neutralization assays. Assays performed on sera collected at 147 and 225 days demonstrated strong type-specific neutralization. Sera from SHIV-HXBc2-infected monkeys neutralized only SHIV-HXBc2. Sera from all SHIV-89.6-infected monkeys neutralized SHIV-89.6 but not SHIV-HXBc2 (Table 2). Surprisingly, sera from all four SHIV-89.6-infected animals neutralized HIV-1 MN, while sera from only two of four SHIV-HXBc2-infected animals had this ability.

Lymphocyte subset changes. To further assess the immunologic responses to these SHIV constructs as well as potential pathologic changes in the animals following infection, changes in circulating lymphocyte subsets were quantitated. The absolute numbers of PBL T-cell subsets and B cells were determined sequentially before and after virus inoculation for each animal. As illustrated in Fig. 6, animals that received either chimeric virus construct had precipitous declines in both circulating T cells and B cells coinciding with the period of plasma viremia. Interestingly, peripheral blood B-cell numbers increased above baseline values in both groups after viral clearance. Absolute circulating $CD4^+$ and $CD8^+$ T-cell counts also recovered coincident with viral clearance and returned to preinoculation levels in the SHIV-HXBc2 group (Fig. 7). However, in the SHIV-89.6-inoculated animals, circulating $CD8^+$ T cells rose above pretreatments levels, resulting in a significant inversion of the PBL CD4/CD8 ratio.

Proliferative T-cell responses to rgp120. To assess the CD4 cell-mediated, virus-specific immune responses during primary infection with these chimeric viruses, in vitro proliferative responses to rgp120 were measured. Within 4 weeks of inoculation, all animals had measurable PBL proliferative responses (Fig. 8). There was significant variability in the magnitude of this response between animals infected with the same virus. This variability was particularly pronounced in the SHIV-89.6-inoculated group of animals: the largest proliferative responses occurred in the PBL of two animals that had the highest peak plasma p27 level and the most prolonged periods of plasma viral antigenemia. However, significant differences between the two groups of infected animals in the time of appearance, magnitude, or duration of the PBL rgp120-specific T-cell response were not evident.

SHIV-specific cytolytic effector cell responses. Cytolytic effector cell responses specific for the *env* gene product of HIV-1 and the *gag* and *pol* gene products of SIVmac were assessed in the PBL and lymph nodes of inoculated monkeys. Virus-specific effector cell activity in PBL was measurable in all animals by 2 weeks following inoculation with either SHIV construct (Fig. 9). Lymphocytes obtained from lymph nodes exhibited similar cytolytic activity at this time point (data not shown). The PBL responses persisted beyond 10 weeks postinfection. One of four animals inoculated with SHIV-HXBc2 failed to develop a measurable cytolytic response to HIV-1 Env, but all developed responses to SIV Gag and, when measured, to SIV Pol. In two of four animals inoculated with SHIV-89.6, cytotoxic responses to SIV Gag were not detected. However, all of these animals showed responses to HIV-1 Env and, when measured, SIV Pol. Thus, neither the presence of the SHIV-specific cytolytic effector cell responses nor their antigen specificity correlated with the extent of virus replication.

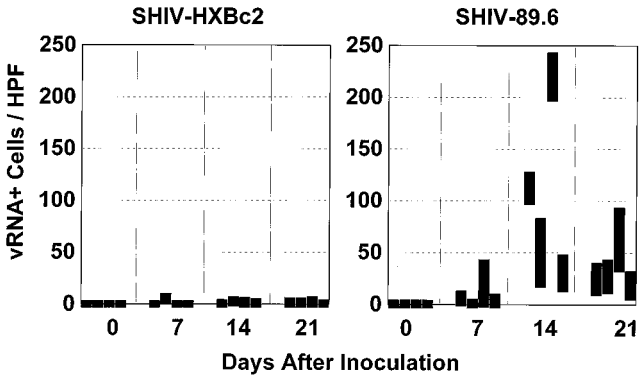

FIG. 5. Number of viral RNA-positive cells observed in lymph nodes analyzed by in situ hybridization in monkeys inoculated with SHIV-HXBc2 or SHIV-89.6. Sections of lymph node biopsies obtained at four time points were hybridized with SIVmac RNA probes as described in Materials and Methods. Each bar represents the range in number of viral RNA positive (vRNA+) cells per high-power field (HPF) observed after analysis of five sections.

DISCUSSION

Chimeric SHIVs provide potentially powerful tools for determining the roles of specific HIV-1 genes in AIDS pathogenesis and for evaluating HIV-1 vaccines. However, previous studies with such chimeric viruses have been disappointing since replication in inoculated macaques has been limited and infection has never been associated with a pathologic consequences (12, 19, 20, 22). Here we report that a SHIV expressing the Env glycoprotein derived from a cytopathic patient isolate of HIV-1 (89.6) exhibited dramatically increased in vivo replicative capacity during primary infection compared with a SHIV expressing HIV-1 Env from a laboratory-adapted virus strain (HXBc2). High levels of SHIV replication were demonstrated both by measuring plasma SIVmac p27 antigen levels

TABLE 2. Neutralizing antibody titers in SHIV-infected macaques

Macaque	Days postinfection	Neutralizing antibody titer to:		
		SHIV-HXBc2	SHIV-89.6	HIV-1 MN
SHIV-HXBc2 infected				
42	147	218	<20	68
471	147	245	<20	<24
149	225	172	<20	24
441	225	70	<20	<24
SHIV-89.6 infected				
483	147	<20	1,105	104
545	147	<20	101	67
123	225	<20	1,962	387
504	225	<20	2,141	32

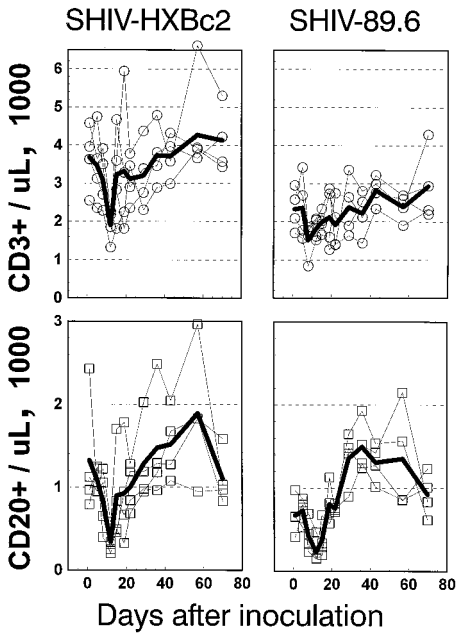

FIG. 6. Changes in peripheral blood T- and B-cell numbers following inoculation with SHIV-HXBc2 or SHIV-89.6. Lymphocytes were quantitated and phenotyped by using monoclonal antibodies that recognized rhesus monkey CD3 (T cells) or CD20 (B cells). Bold lines represent mean values of all animals tested.

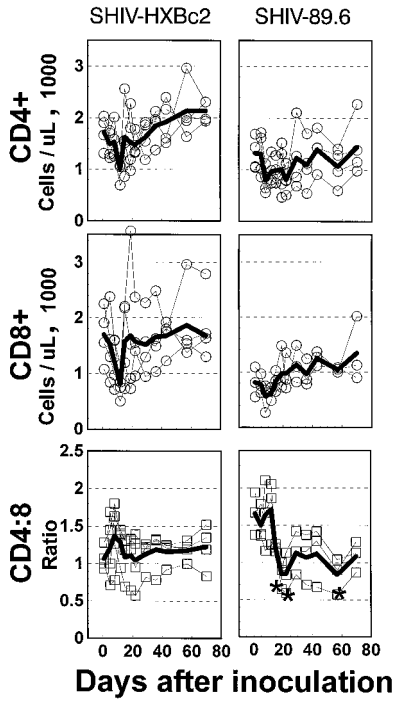

FIG. 7. Changes in peripheral blood T-cell subsets following inoculation with SHIV-HXBc2 or SHIV-89.6. T-cell subsets were quantified and ratios were determined by using antibodies recognizing rhesus monkey CD3 and CD4 or CD3 and CD8. Bold lines represents mean values of all animal tested. Asterisks indicate a significant decrease from the preinoculation mean value.

and by assessing virus replication in situ in lymph nodes. In fact, the levels of SHIV-89.6 replication during the first weeks of primary infection of rhesus monkeys were comparable to those of SIVmac251 (32).

The difference in in vivo replicative capacities of SHIV-HXBc2 and SHIV-89.6 was not predicted by the in vitro replication of these two viruses on rhesus monkey PBL. Paradoxically, SHIV-HXBc2 replicated well in vitro in monkey PBL, while SHIV-89.6 replicated poorly; neither chimeric virus was macrophage tropic. These observations suggest that caution should be exercised in extrapolating the in vivo pathogenic potential of HIV-1 isolates from in vitro manifestations of infection.

Certain immunopathologic changes occurred after infection with both SHIV-HXBc2 and SHIV-89.6. Infection with both viruses was associated with a marked lymphopenia at 2 weeks postinoculation, coinciding with the initial burst of virus replication in all monkeys. An increase in circulating B cells over the preinoculation levels was also seen in monkeys inoculated with either virus. This B-cell lymphocytosis corresponded with the development of germinal centers within the lymph nodes of all animals. It will be interesting to determine whether the B-cell response represents a clonal, antigen-specific response, a polyclonal nonspecific response to lectin-like properties of the viruses, or even a V-gene family-restricted B-cell response to superantigen-like components of the viruses.

In recent studies, SIV from African green monkeys has been shown to induce an AIDS-like syndrome in pig-tailed macaques but not in rhesus macaques or African green monkeys (10). This species-specific variation in pathogenicity correlated with the degree of virus replication in the host, with high virus replication predicting pathogenicity. Although the monkeys in the present study have not been infected long enough to determine whether SHIV-89.6 will induce an immunodeficient state, it is interesting that T-cell subset perturbations occurred only in animals infected with SHIV-89.6. While absolute CD4 counts recovered following lymphopenia in these animals, their CD4/CD8 ratios inverted following clearance of primary viremia, largely as a result of an absolute increase in circulating $CD8^+$ T cells. Similar persisting T-cell shifts are observed following primary infection of humans with HIV-1 and macaques with pathogenic SIVmac (2, 4, 32). The molecular histology studies also detected significant trapping of virus within lymph node germinal centers in SHIV-89.6-inoculated monkeys. Since this finding correlated with the appearance of envelope-specific antibodies, such trapping may represent anti-

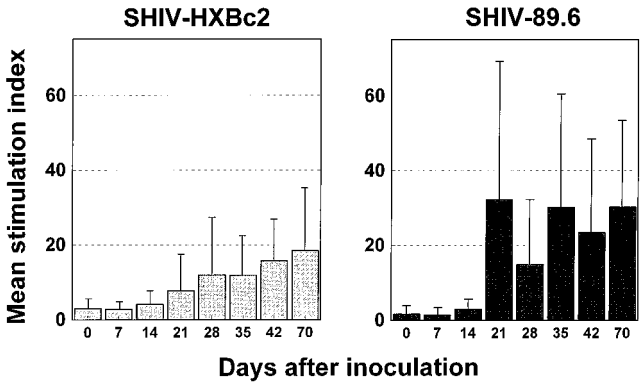

FIG. 8. Monkeys inoculated with either SHIV-HXBc2 or SHIV-89.6 develop proliferative responses to HIV-1 gp120. Results are given as stimulation index calculated from the mean counts per minute of [^{3}H]thymidine incorporation in triplicate antigen-stimulated (1 μg of HIV-1 gp120 per ml) wells divided by the mean counts per minute in control wells containing medium alone. Each bar represents the mean stimulation index ± standard deviation for four animals.

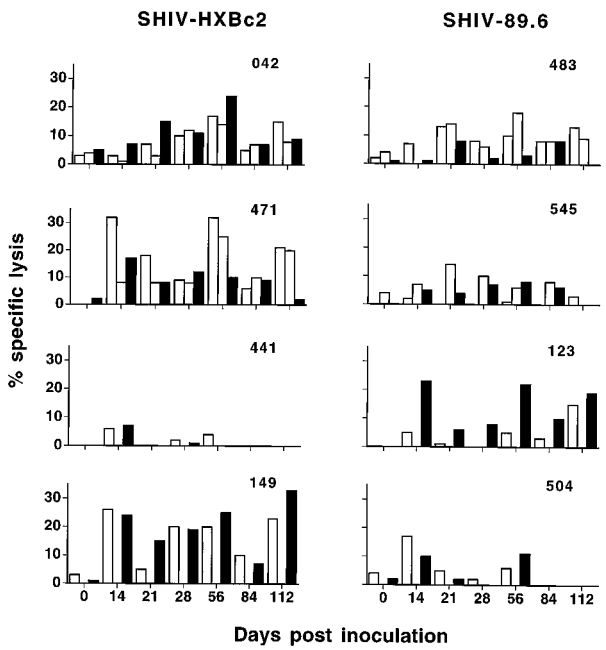

FIG. 9. Monkeys inoculated with either SHIV-HXBc2 or SHIV-89.6 develop both HIV-1- and SIVmac-specific cytotoxic effectors cell responses. PBL obtained from monkeys at the indicated time points were stimulated with concanavalin A and expanded in interleukin-2-supplemented medium. Lytic activity of those cells was determined against autologous B-lymphoblastoid cell lines infected with wild-type or recombinant vaccinia virus expressing HIV-1 Env (■), SIVmac Gag (▨) or SIVmac Pol (□; upper four panels only). Percent specific lysis was calculated by subtracting percent ^{51}Cr release of wild-type vaccinia virus-infected target cells from the respective values obtained with viral antigen-expressing targets.

body-promoted opsonization of virus and the retention of this opsonized virus by complement receptors on the surface of follicular dendritic cells (14, 24, 26). Virus trapped in this way could be a persisting reservoir for transmitting infection to $CD4^+$ lymphocytes residing or trafficking through lymphoid organs as a mechanism contributing to disease pathogenesis (8, 36, 38). These observations point to the possibility that SHIV-89.6 can induce an AIDS-like disease in chronically infected macaques.

The differences in replicative capacity of these two chimeric viruses could not be explained by a quantitatively different virus-specific immune response to the viruses in the infected animals. Both groups of monkeys developed HIV-1 envelope-specific antibodies in similar time frames and with type-specific neutralizing activity. In addition, proliferative responses to rgp120 and virus-specific cytolytic responses to both HIV-1 and SIVmac gene products were similar in the two groups. This finding suggests that the increased in vivo replicative capacity of SHIV-89.6 may be a biological phenomenon attributable to the 89.6 Env.

The importance of the envelope glycoproteins in the replicative capacity and pathogenicity of the AIDS virus has been demonstrated in other studies. Hirsch et al. have shown that the V3-loop analog of SIVmac plays an important role in the pathogenicity of SIVmac in macaques (11). Luciw et al., in a comparative in vivo study of SHIVs constructed with *env* genes of either a T-cell-tropic or macrophage-tropic HIV-1 isolate, demonstrated envelope-determined differences in the replicative capacity of the chimeric viruses (22). In the present study, the chimeric viruses constructed with the patient isolate *env* gene 89.6 replicated substantially more efficiently than the SHIV constructed with the *env* gene of the laboratory isolate of HIV-1, HXBc2 during primary infection. These results indicate that sequence differences within *env* may have a marked effect on the replicative capacities of SHIVs in nonhuman primates during primary infection. Analogous changes occurring naturally in HIV-1 *env* may similarly accelerate HIV-1 replication during primary infection in humans.

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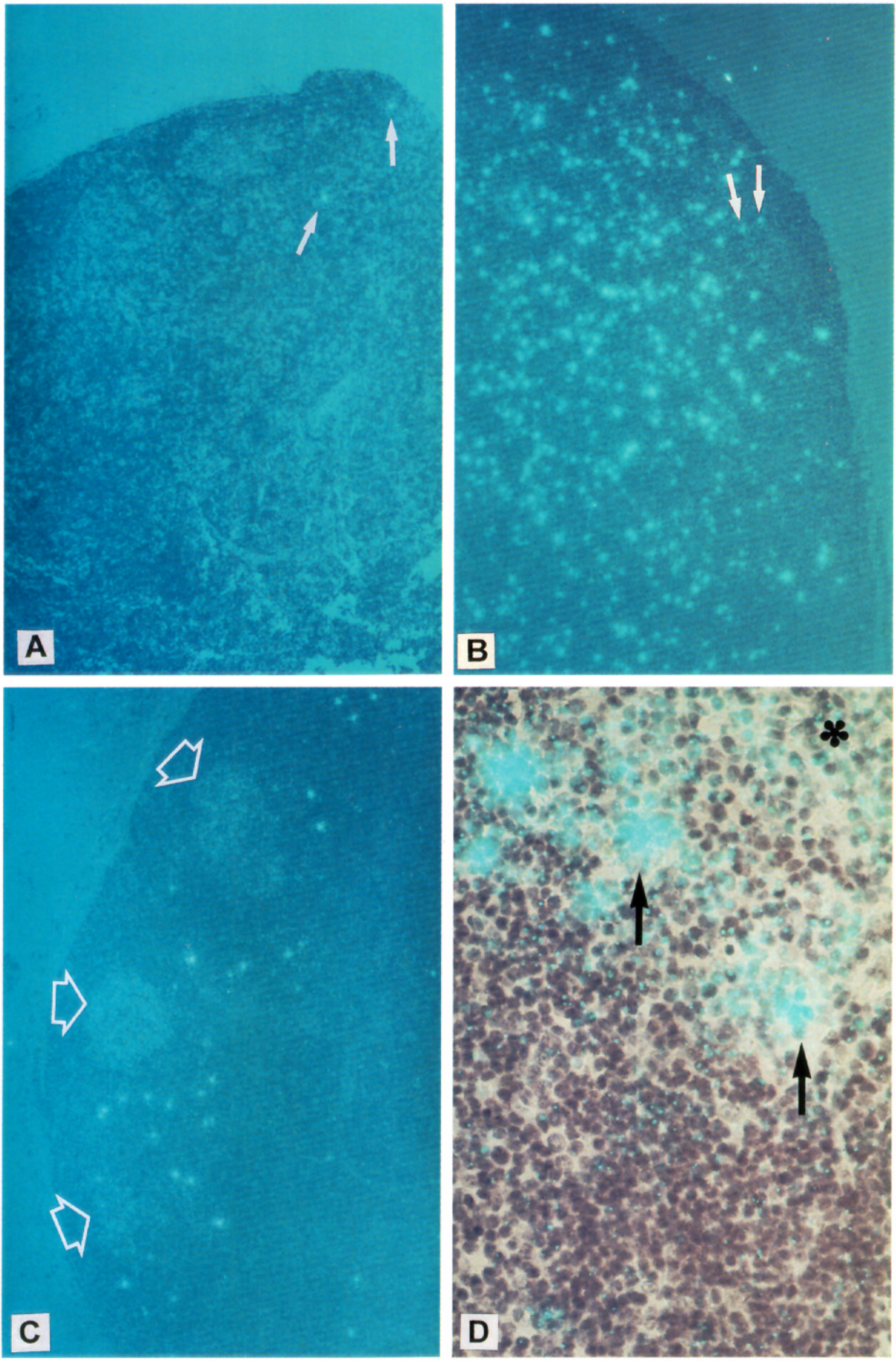
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