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Syphilis Superinfection Activates Expression of Human Immunodeficiency Virus I in Latently Infected Rabbits

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Superinfection of latently human immunodeficiency virus (HIV)-infected rabbits with either Treponema pallidum ***or Shope fibroma virus (SFV) activates HIV expression. In addition, HIV-infected rabbits demonstrate prolonged cutaneous lesions (chancres) after intracutaneous challenge with*** T. pallidum, ***the causative agent of syphilis. Rabbits were infected by intravenous inoculation of*** 3×10^7 ***human T-cell lymphotrophic virus type III (HTLV-III)/B10 (HIV-1)- infected H9 (human) cells. Five weeks after initial infection, integrated HIV-1–specific DNA sequences were detected in the DNA of the peripheral blood lymphocytes of only one of eight rabbits using polymerase chain reactions (PCR); human DNA could not be detected at this time. Furthermore HIV infection could not be demonstrated by either seroconversion or PCR during the next 6 months. All HIV-infected rabbits remained clinically healthy and had normal white blood cell counts. Six months after HIV infection, four HIV-infected and two noninfected controls were superinfected with*** 10^6 T. pallidum ***in eight skin sites in the shaved skin of the back, and four infected and two control animals were challenged with an intradermal injection with SFV. After infection with either syphilis or SFV, the DNA from the white blood cells of all eight HIV-infected rabbits contained HIV sequences, and HIV sequences were demonstrated in dermal mononuclear cells of the syphilitic lesions by*** in situ ***hybridization. The SFV-induced tumors were rejected normally in the HIV-infected rabbits, but four of the four rabbits challenged with*** T. pallidum ***had delayed development of cutaneous lesions and three of four demonstrated larger and more prolonged lesions. White blood counts, mitogen responses, and interleukin-2 production remained within normal limits, and seroconversion for HIV was not detected. Three of four rabbits in a second group, challenged with*** T. pallidum ***4 months after HIV-inoculation, also had delayed healing of syphilitic lesions. These results indicate that latent HIV-infection of rabbits may be activated by immunostimulation and that latently HIV-infected rabbits have impaired delayed hypersensitivity reactions. It is hypothesized that true latent HIV-infection in the rabbits is in monocytes and postulated that further immunostimulation may produce infection of lymphocytes and activation of disease. (Am J Pathol 1991, 138:1149–1164)***

One of the highest priorities in acquired immune deficiency syndrome (AIDS) research is to develop an animal model to study the pathogenesis of the disease and provide a means for testing vaccination or therapy protocols.[1–4] However the available animal models of AIDS, primates and immune-deficient mice reconstituted with human lymphoid cells, leave much to be desired. The most extensively studied model is that of simian immunodeficiency virus in macaques (SIVmac).[4–7] However an animal model to study human immunodeficiency virus (HIV) infection directly would be preferable to study of a related virus. Infection of primates with HIV has been possible, but the models have been expensive and not very useful.

Clearly a small-animal model for AIDS would be more desirable. Several mouse models using either transgenic mice carrying complete copies of HIV genes[8] or severe combined immunodeficient mice reconstituted with human lymphoid cells[9–11] and infected with HIV have been created. Although each of these models has great promise in the study of certain aspects of AIDS infections, they are highly artificial and harbor many other viruses, such as Epstein–Barr virus. Considerable care will need to be

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taken in extrapolating any results on pathogenesis or effects of vaccination or treatment of these mice to the human situation.

Although infection of rabbits with human retroviruses, both human T-cell lymphotrophic virus type I (HTLV-1)[12–16] and HIV-1,[17–19] has been reported, many workers in the field apparently are not convinced that rabbits can be infected successfully with HIV. Rabbits inoculated with either cell-free HIV-1 or HIV-1–infected H9 cells demonstrated a persistent infection with seroconversion and positive viral infectivity of H9 indicator cells but no disease manifestations.[18] Superinfection of HTLV-1–infected rabbits with HIV-1 using an irradiated HIV-infected cell line resulted in seroconversion and positive blood cultures for HIV virus. Although no overt disease was seen, '. . . certain gross and microscopic findings not unlike human AIDS'[19] were found. We now report preliminary experiments demonstrating activation of expression of latent HIV-1 infection of rabbits by superinfection with *Treponema pallidum*, the causative agent of syphilis, or with Shope fibroma virus (SFV). In addition, an immune deficit is revealed in three of four HIV-infected rabbits after challenge with *T. pallidum* in two separate experiments.

Materials and Methods

Rabbits

Outbred New Zealand white male rabbits (weighing 3 to 4 kg), obtained from Ray Nichols Rabbitry (Houston, TX), were housed individually in the certified central facility (biosafety level 2) of the medical school at the University of Texas Health Science Center at Houston, Texas. Before experimental infection, the rabbits were healthy and prebleedings were negative for antibodies to HIV-1 or *T. pallidum*. All animal protocols were approved by the Animal Welfare Committee and the Biohazard Committee of our institution.

Cells

Peripheral blood lymphocytes (PBL) of rabbits were prepared from blood obtained from the ear artery after defibrination and sedimentation in an equal volume of 3.5% pig skin gelatin–phosphate-buffered saline (PBS) for 30 minutes at 37°.[20] Red blood cells were lysed osmotically and recovered cells were used as PBL. Total white blood cells (WBC) were prepared from the buffy coat of anticoagulated blood.

Human immune virus-1–infected and uninfected H9 cells were obtained from Dr. Tse Wen Chang's laboratory (Baylor College of Medicine, Houston, TX) through the assistance of Randi Zicht in Dr. R. C. Gallo's laboratory (Laboratory of Tumor Cell Biology, NIH). The HIV-1 is the HTVL-III/B10 strain. These cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 15% fetal calf serum (FCS). Human immune virus-1–infected H9 cells continuously released viral particles *in vitro*, as evaluated periodically by reverse transcriptase activity and electron microscopic identification of virus.

HIV-1 Infection

Rabbits were injected intravenously with 3×10^7 HIV-1–infected H9 cells showing peak reverse transcriptase activity.

DNA Isolation

Total cellular DNA was isolated according to standard methods.[21] Rabbit PBLs, WBCs, or HIV-infected H9 cells at the density of approximately 5×10^6 to 2×10^7 cells/ml were lysed in 100 mmol/l (millimolar) TRIS, pH 8.0, 10 mmol/l ethylenediamine tetraacetic acid (EDTA), and 1% sodium dodecyl sulfate (SDS), followed by proteinase K treatment (100 μg/ml) for 3 hours at 37°C. DNA was extracted with an equal amount of phenol/chloroform and dialyzed extensively with TRIS buffer containing EDTA (TE). The resulting suspension was treated with DNAse-free RNAse for 2 hours at 37°C to digest the RNA. After extraction with phenol/chloroform, the suspension was dialyzed with TE buffer.

Gene Amplification by Polymerase Chain Reaction (PCR)

The oligonucleotide primers and probe specific for the gag region of HIV-1 were SK 38/39 and SK 19, respectively.[22,23] The SK38 and SK 39 for HIV-1 are 28 mers and 29 mers, respectively, and are 57 base pairs apart. An amplified fragment of 114 base pairs thus is expected. The human alpha-satellite sequences are composed of a family of closely related 171 base pair random repeats.[11] The SAT 20/26 primers are specific for the 5′ and 3′ ends of the consensus repeat sequence. These oligonucleotides were synthesized on a DNA synthesizer (Cyclone, Biosearch Inc., San Rafael, CA) by Dr. Brian Knoll in our department and purified by Nensorb column (DuPont/NEN, Boston, MA). The PCR was performed with 'Gene Amp DNA Amplification' reagents kit and 'DNA Thermal Cycler' from Perkin Elmer Cetus (Norwalk, CT). Briefly, 0.5 to 1.0 μg of DNA extracted from HIV-infected H9 cells or rabbit PBLs or peripheral blood

mononuclear cells (PBMC) was amplified through 35 cycles. Each cycle consisted of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for 90 seconds. The concentration of nucleotides, buffer composition, and concentration of primers were used as recommended by Cetus. One unit of thermus aquaticus (Tag) enzyme in a volume of 100 μl of mixture was used. The DNA extracted from HIV-1–infected H9 cells was used as positive control. Negative controls were DNA derived from PBL or total white cells of HIV-1–uninfected rabbits. Human DNA was probed in the same samples using rabbit WBC DNA and PCR primed with ACTTCTCAGAACTTCTTTCTCAT (sense) and TTTTATATGAAGATATTCCC (anti-sense) and probed with p^{DP97}, a human alpha-satellite probe (lot 57606, ATCC, Rockville, MD) specific for sequences on chromosome 17.[11,24]

Southern Blot Analysis

An aliquot of 10 l of the PCR product was electrophoresed on 1.8% agarose gels in TRIS-acetate-EDTA (TAE) buffer at pH 8.0. The gel was stained with ethidium bromide and photographed. The DNA was transferred onto nitrocellulose (BA 83, Schleicher & Schuell, Keene, NH) according to standard procedures.[21] The nitrocellulose membrane with immobilized DNA was hybridized with specific probes labeled with isotopes. ^{32}p end-labeled SK 19 probe was used to detect HIV-1–specific DNA. The human alpha-satellite probe, (p^{DP97}) labeled with ^{32}p by random priming (Pharmacia, Piscataway, NJ), was used to detect human DNA sequences. The membranes were rinsed three times at 55°C in 2 × SSC containing 0.1% SDS, and the autoradiograms developed overnight at −70°C with intensifying screens.

Serologic Analysis

The antibody response of HIV-1–infected rabbits was evaluated by Western blotting. Nitrocellulose strips containing HIV-1–specific proteins were prepared in our laboratory from viral lysates, according to the method described.[25] Released viral particles in the supernatant of HIV-1–infected H9 cells, showing peak reversed transcriptase activity, were precipitated overnight at 4°C with 30% polyethylene glycol 6000 in PBS. The precipitates were pelleted by centrifugation at 1200*g* for 45 minutes and dissolved in the appropriate amount of solution containing 50 mmol/l Trishydrochloride (pH 8.0), 0.5% Triton x-100, 5% glycerol, 0.8 mol/l (molar) NaCl, and 2 mmol/l dithiothreitol (Boehringer Mannheim, Indianapolis, IN). The solubilized viral lysate was mixed with equal volumes of SDS-polyacrylamide gel electrophoresis sample buffer and boiled for 5 minutes. The samples were subjected to electrophoresis on 10% polyacrylamide gel and the proteins electrotransferred onto nitrocellulose paper. Nitrocellulose strips derived from the culture supernatant of HIV-uninfected H9 cells also were prepared as negative controls. Heat-inactivated serum samples from HIV-1–infected and uninfected rabbits were diluted 1:100 and used as the primary antibodies for the immunoperoxidase staining of the electroblots. Peroxidase-conjugated goat anti-rabbit immunoglobulin (1:2000 dilution, Cappel-Cooper Biomedical, Inc., West Chester, PA) was used as the second antibody and diaminobenzedine (Sigma Chemical Co., St. Louis, MO) was used as color reagent. Sera derived from AIDS patients were used as positive controls. The second antibody used to detect human Ig was also raised in a goat. Serum samples were sent coded to Dr. James Carlson at the Retrovirology Laboratory at University of California at Davis for independent analysis.

Serum was collected from each rabbit before injection (day 0) and on days 15 and 64 after superinfection with *T. pallidum*. Veneral Disease Research Laboratory (VDRL) slide flocculation tests for syphilis were used to evaluate the serologic response to *T. pallidum* (Manual of Tests for Syphilis. USDHEW, National Center for Disease Control, Atlanta, GA, 1969). Heated serum (0.05 ml) was transferred into one ring of a paraffin-ringed slide; 20 l of antigen suspension was then added to the serum. The slides were rotated for 4 minutes at 180 rpm and read microscopically with 10 × ocular and a 10 × objective, immediately after rotation.

Superinfection of Rabbits with T. Pallidum *or SFV*

The Nichols strain of *T. pallidum* was maintained by intratesticular passage in rabbits and the treponemes were extracted from rabbit testes according to the method described.[26] For the superinfection, 1×10^6 *T. pallidum* in 0.1 ml treponemal suspension was injected intradermally into eight sites on the clipped backs of four HIV-1–infected and two uninfected rabbits. Each inoculation site was examined daily for 3 weeks and three times a week for the next 5 weeks for the development of the syphilitic lesions. To quantitate the extent of lesion development, the diameter of each of the eight lesions on each rabbit was measured and the average diameter was calculated. For challenge with SFV, 10^6 plaque-forming units of SFV in 0.1 ml was injected intradermally into each of eight sites on the clipped backs of four HIV-1–infected and two uninfected rabbits in two separate experiments.[27]

Biopsy of Rabbits

Before biopsy, rabbits were sedated by intramuscular injection (0.5 ml/kg body weight) of a 1:1 (vol/vol) mixture of Ketaset (Ketamine HCl, 100 mg/ml, Parke-Davis, Morris Plains, NJ) and Rompun (20 mg/ml, Miller Vet Supply, Fort Worth, TX). Biopsies were taken from one control and one HIV-infected rabbit 15 and 20 days after superinfection with *T. pallidum* or SFV. Sections from the biopsies were fixed in neutral-buffered formalin and processed routinely for embedding in paraffin and staining with hematoxylin and eosin (H&E).

Visualization of T. pallidum

The Bosma-Steiner staining procedure for spirochaetes[28] was used to demonstrate the presence of *T. pallidum* in syphilitic lesions. Deparaffinized sections of formalin-fixed tissue were incubated at 37°C for 1.5 hours in a solution of 1% amylase (Sigma, #3176) in distilled water. The sections were rinsed for 5 minutes in absolute ethanol and 5 minutes in uranyl nitrate-mastix solution (3 g uranyl nitrate [Fluka #94270] dissolved in 70 ml 100% ethanol, to which is added 30 ml of 10% [w/v] gum mastic) then rinsed with distilled water. The sections then incubated at 60°C for 1 hour in a solution of 1% silver nitrate (Sigma, S-181) in distilled water. The sections were again rinsed for 5 minutes in 100% ethanol and 5 minutes in ethanol-mastix solution (4 g gum mastic dissolved in 100 ml 100% ethanol). Finally the sections were incubated at 60°C for 1.5 hours in a solution of 5 g pyrocatechol (Kodak #604, Eastman-Kodak, Rochester, NY) dissolved in 100 ml distilled water. After dehydration in ethanol, acetone, and xylene, coverslips were mounted on the slides. *T. pallidum* stained by this procedure appears black on a yellow background.

Mitogen-induced Cell Proliferation

Mitogen-induced blastogenesis of PBL derived from HIV-infected and -uninfected rabbits was evaluated after *T. pallidum* superinfection. Two $\times 10^5$ cells/well in a total volume of 0.2 ml RPMI-1640 medium containing 10% FCS was incubated with the three dilutions of concanavalin A (Con A; Sigma) for 72 hours in 96-well flat-bottom microtiter plates (Costar, Cambridge, MA). The cultures were pulsed with 0.1 μCi/well of [^{125}I]-iododeoxy-uridine (IUdr; ICN Radiochemicals, Irvine, CA) for the last 16 hours, and were harvested onto glass fiber filters using an automatic harvester. Blastogenesis was expressed as total IUdr uptake per culture, which was measured on a gamma counter.

Interleukin 2 (IL-2) Production and Assay

Con A-induced IL-2 production also was determined after superinfection. One ml of rabbit PBL suspension containing 1×10^6 cells was incubated with the indicated concentration of Con A for 48 hours at 37°C. The resulting culture supernatants were harvested by centrifugation to remove cells. For the IL-2 assay, the IL-2–dependent murine CTLL-2 line was used, as described.[29] CTLL-2 cells were cultured in 96-well flat-bottom microtiter plates (Costar, Cambridge, MA) at a concentration of 5×10^3 cells/well in a total volume of 0.2 ml RPMI-1640 medium containing 10% FCS and diluted (1:4) test samples. After 44 hours of incubation at 37°C, the plates were pulsed for 4 hours with 0.1 μCi of [^{125}I]Udr. Duplicate cultures were set up for each individual samples. The IL-2 production of each rabbit was expressed as the total [^{125}I]Udr uptake (cpm) by the cultured cells.

Proliferation of Treponemal Antigen-specific PBLs

Peripheral blood lymphocytes were prepared from both HIV-1–infected and noninfected rabbits. Sonicated treponemal antigen for *in vitro* stimulation of lymphocytes was obtained by extraction of infected rabbit testes in sterile normal saline and removal of gross tissue debris as previously described.[26] Organisms were washed three times with sterile 75 mmol/l sodium nitrate counted by darkfield microscopy, and suspended in sterile saline at 10^9 organisms per milliliter. The preparation was sonicated on ice for 2 minutes using a Tekma Sonic Disrupter (Cincinnati, OH) set on the highest power level. Aliquots of 1 ml were made and stored at −20°C until reused. To determine treponemal antigen-induced lymphoblastogenesis, 2×10^5/well of PBL, in a volume of 200 μ1 RPMI-1640 medium supplemented with 10% FCS, was cultured with or without 10 μl sonicated *T. pallidum* (10^7 organisms) for 4 to 7 days at 37°C with 5% CO_2. Duplicate cultures were set up for each individual rabbit. All the cultures were pulsed with 0.1 μCi of [^{125}I]Udr (ICN Radiochemicals) for the last 16 hours, and harvested onto glass fiber filters. The treponemal antigen-induced lymphoblastogenesis was expressed as total [^{125}I]Udr uptake (cpm), which was assessed by a gamma counter.

In Situ Hybridization

In situ hybridization for HIV sequences in the syphilitic lesions of HIV-infected and control rabbits was performed using tat and nef riboprobes. An antisense riboprobe to K14 keratin was used as a positive control on all

skin biopsies. The [^{35}S]-labeled RNA was transcribed from T7 and Sp6 promoter regions (approximately 1090 bp insert subcloned into pSP65 vector, provided by Elaine Fuchs, University of Chicago). Specific activity of the keratin riboprobe was 1.43 × 10^6 cpm/μl. Riboprobes for the tat and nef regions were used to detect HIV sequences.[30] Two fragments of HIV-1 DNA representing the tat portion (0.65-kb insert from HIV clone pNIG-G, provided by Larry Donehower) and the nef portion (2.3-kb insert from HTLV-III clone HxB2, provided by David Volsky, University of Nebraska) of the HIV genome were subcloned into pGEM and pSP73, respectively. The [^{35}S]-labeled RNA was transcribed from T7 and Sp6 promoter regions. Sp6 or T7 RNA polymerase (Promega Biotec) was used to generate either antisense or sense RNA transcripts. The specific activity of tat antisense and sense control probes was 2.55 to 3.14 × 10^6cpm/μl. The specific activity of nef antisense and sense control probes was 1.4 to 3.44 × 10^6 cpm/μl.

Four-micron–thick sections of 4% paraformaldehyde-embedded skin biopsies were placed on aminoalkylsilane–treated slides[31,32] and hybridization was performed as described by Harper et al.[33] Following xylene treatment and rehydration, the slides were treated with proteinase K, acetylated, treated with 0.1 mol/l TRIS-HCl (pH 7.0) and 0.1 mol/l glycine, and then hybridized with 10^7 counts/minute of the 35S-labeled HIV antisense or sense (negative controls) riboprobes. Autoradiography was performed using Eastman Kodak NTB2 emulsion exposed at 4°C for 2 days, developed in Dektol developer (Kodak, Rochester, NY), and stained with giemsa or H&E. Sections were not subjected to denaturing before hybridization to ensure that the probe detected RNA and not DNA, which would remain double stranded without denaturation. The slides were examined first by conventional light microscopy to locate areas of suspected hybridization, followed by dark field, and then by confocal microscopy[34,35] at the Integrated Microscopy Resource in Madison, Wisconsin, using a BioRad MRC-500 confocal laser scanning microscope. Scanning of the specimen was performed by moving the laser beam through the specimen at 0.5-μ increments, producing an optical section of the tissue with little degradation of image quality. Positive hybridization is confirmed if the silver grains remained in the confocal image above the level of the nonspecific background following the optical sectioning of the specimen.

Results

Detection of HIV-1 DNA in PBLs of Infected Rabbits

The infection of rabbits by HIV-1 was first evaluated by PCR to detect the integrated DNA in PBLs. As shown in a representative Southern blot analysis using HIV-1–specific SK 19+ probe, only one of the eight infected rabbits gave a positive signal of HIV-1 infection 5 weeks after infection (Figure 1). No human DNA sequences were found at this time (Figure 2). However each of the eight infected rabbits were positive for HIV DNA when tested 9 months after infection (and 3 months after *T.*

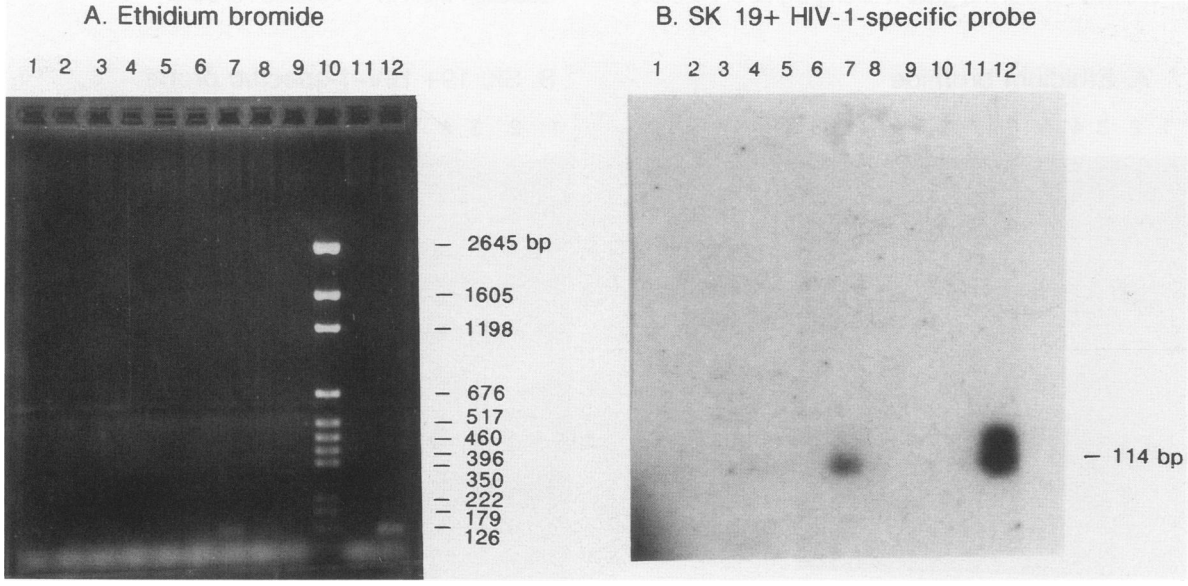

Figure 1. *Southern blot analysis of PCR amplified DNA from PBLS 5 weeks after HIV inoculation detects HIV in only one of eight rabbits.* **A:** *Ethidium bromide-stained agarose gel of PCR products.* **B:** *Southern blot after hybridization with SK19+ probe. Lanes 1–8: DNA from HIV-infected rabbits #400–407; lane 9: uninfected rabbit; lane 10: molecular weight markers; lane 11: DNA from 1 HIV-infected H9 cell/$10^6$6 normal rabbit cells; and lane 12: DNA from HIV-infected H9 cells. Only the DNA from rabbit #406 contains HIV sequences.*

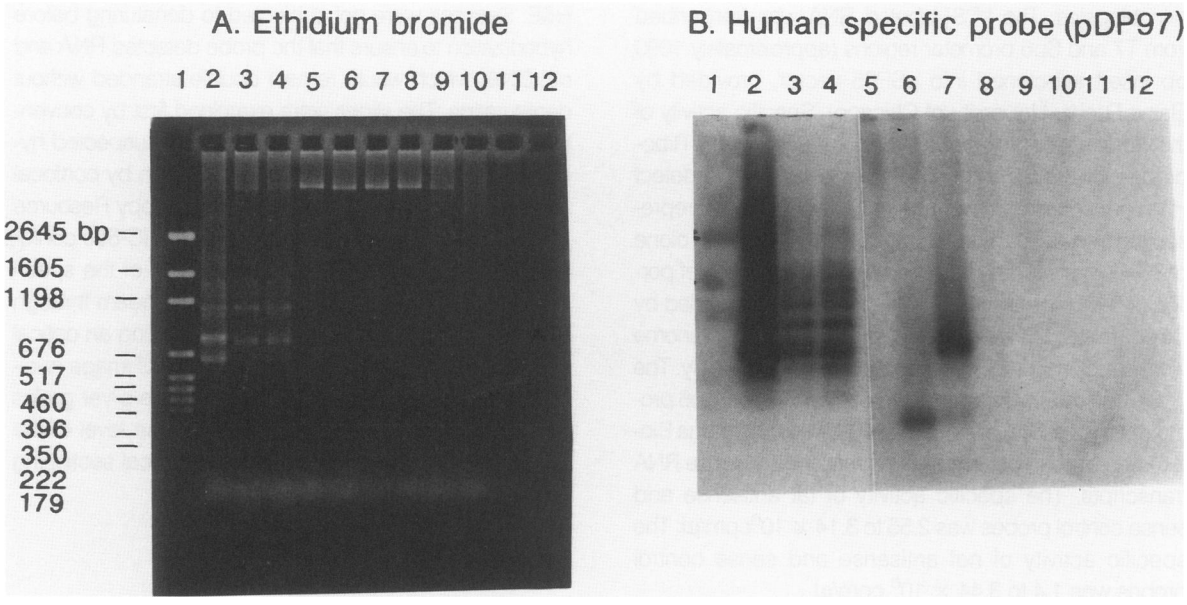

Figure 2. *Human DNA is not detected in DNA from rabbit PBLS 5 weeks after infection using PCR and human DNA primer pair SA20 and 26.* **A:** *Ethidium bromide-stained gel of PCR products.* **B:** *Southern blot using probe specific for human satellite DNA on chromosome 17 (P^{DP97}, ATCC). Lane 1: Molecular weight markers; lane 2: DNA from HIV-infected H9 cells; lane 3–9: DNA from 1/10, 1/20, $1/10^2$, $1/10^3$, $1/10^4$, $1/10^5$, $1/10^6$ HIV-infected H9 cells mixed with normal rabbit peripheral blood white cells; lane 10: DNA from rabbit 1286 (control); lane 11: DNA from rabbit 406 (5 weeks HIV-infected); lanes 2–4: Exposed for 20 minutes; and lanes 5–11 exposed for 68 hours. The PCR was primed with SA20, ACTTCTCAGAACTTCTTTCTCAT (sense) and SA26, TTTTATATGAAGATATTCCC (anti-sense) and the products probed with p^{DP97}, a human alpha-satellite probe specific for sequences on chromosome 17 (23,24). Human DNA was not detected in any of the rabbits 5 weeks after inoculation with HIV-positive H9 cells, including rabbit 406 (positive for HIV).*

pallidum superinfection) (Figure 3). Human satellite DNA was not detected in these samples (Data not shown). Analysis of mixtures of HIV-infected H9 cells and uninfected rabbit white blood cells indicated that one HIV-infected cell in 10^5 uninfected cells could be detected (Figure 4). The positive sample prepared from the HIV-1–infected H9 cell line gave a strong signal of infection, revealed by both ethidium bromide staining and Southern blot analysis, whereas the DNA from HTLV-1–infected rabbit lymphoblastial T cells (RLT-P)[16] was negative (data not shown). The positive reactivity of DNA samples from infected rabbits indicates that rabbits can be infected successfully with HIV-1 by the intravenous inoculation with HIV-1–infected H9 cells.

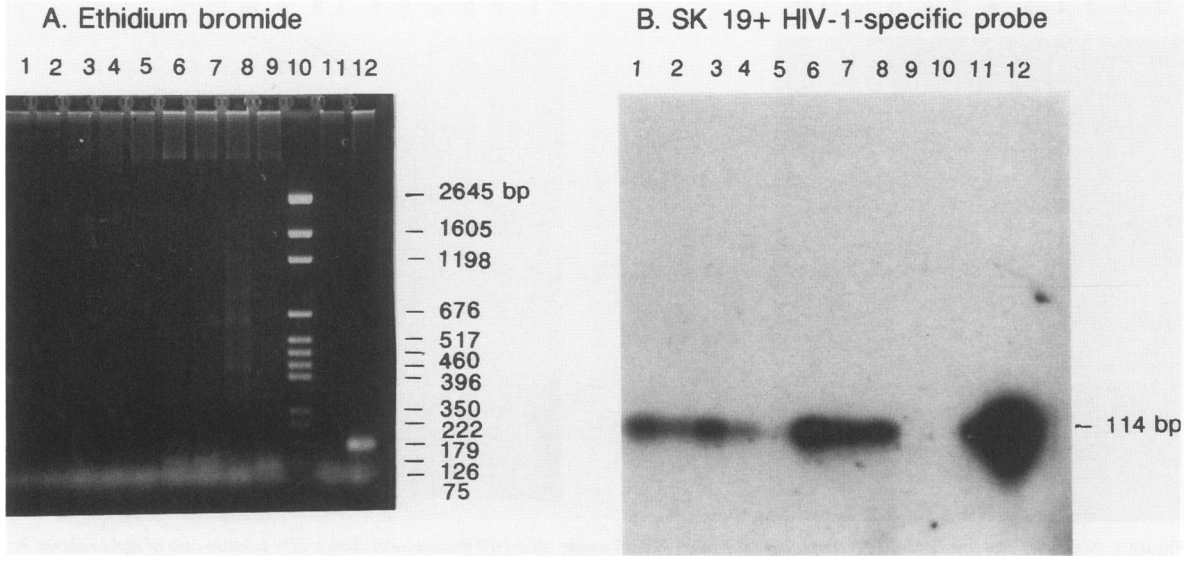

Figure 3. *HIV sequences are detected in the DNA of the peripheral blood white cells of all eight HIV-infected rabbits 9 months after inoculation of HIV-infected H9 cells.* **A:** *Ethidium bromide-stained gel of PCR products.* **B:** *Southern blot using SK19+ probe (exposed 19 hours). Lanes 1–8: DNA from HIV-infected rabbits (#s 400–407); lane 9: DNA from uninfected rabbit (# 1707); lane 10: Molecular weight markers; lane 11: DNA from 1 HIV-infected H9 cell mixed with 10^5 normal rabbit peripheral blood white cells; and lane 12: DNA from HIV-infected H9 cells.*

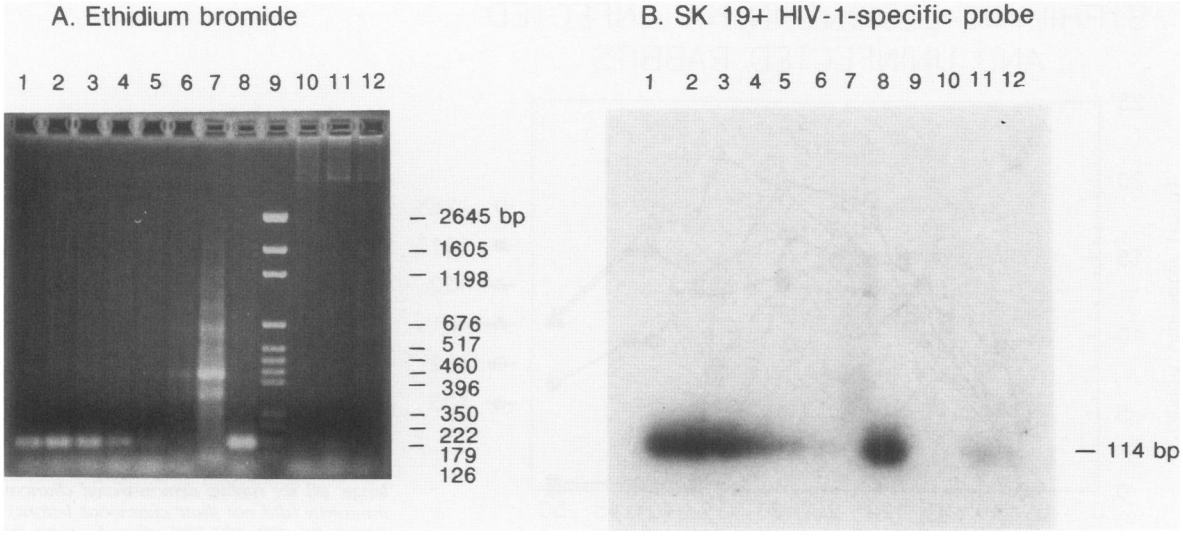

Figure 4. *PCR for HIV sequences detects one HIV-infected H9 cell in 10^5 normal rabbit peripheral blood white cells.* **A**: *Ethidium bromide-stained cells.* **B**: *Southern blot with SK 19+ HIV-specific probe (exposed 19 hours). Lanes 1–7: DNA from mixtures of 1 HIV-infected H9 cells in 10, 20, 10^2, 10^3, 10^4, 10^5, and 10^6 uninfected rabbit white cells; lane 8: DNA from 100% infected H9 cells; lane 9: Molecular weight markers; lane 10: DNA from white cells of uninfected rabbit (# 1701); and lanes 11,12: DNA from white cells of HIV-infected rabbits (#s 405 and 406) 9 months after infection.*

Serologic Analysis of Infected Rabbits

Serum samples collected from each rabbit 5, 21, and 26 weeks after HIV infection, as well as on days 0, 15, and 64 after *T. pallidum* challenge were analyzed by immunoblotting using nitrocellulose strips containing HIV-1 proteins, prepared from HIV-1 viral lysate. Positive controls from AIDS patients revealed the expected levels. These sera were sent along with control sera in coded vials to Dr. James Carlson (U.C. Davis) for confirmation of HIV antibody, using positive rabbit anti-HIV as controls. As shown in Figure 5, each of the 8 HIV-1–injected rabbits reacted with a band consistent with p51, although weakly. The significance of this reactivity (to reverse transcriptase) is not clear. Sera from each of the infected rabbits also gave a band at p24, but this band also was seen in control sera from uninfected rabbits. Serologic analysis of rabbits after *T. pallidum* or SFV challenge did not reveal additional reactivity.

Progressive and Prolonged Cutaneous Syphilitic Lesions in HIV-1–Infected Rabbits

HIV-1 infection in humans usually causes an irreversible immunosuppression by selectively infecting and ultimately lysing CD4-positive T lymphocytes, resulting in a immune defect that renders the body highly susceptible to 'opportunistic' infections and neoplasms.[36] However such profound immunodeficiency caused by HIV-1 infec-

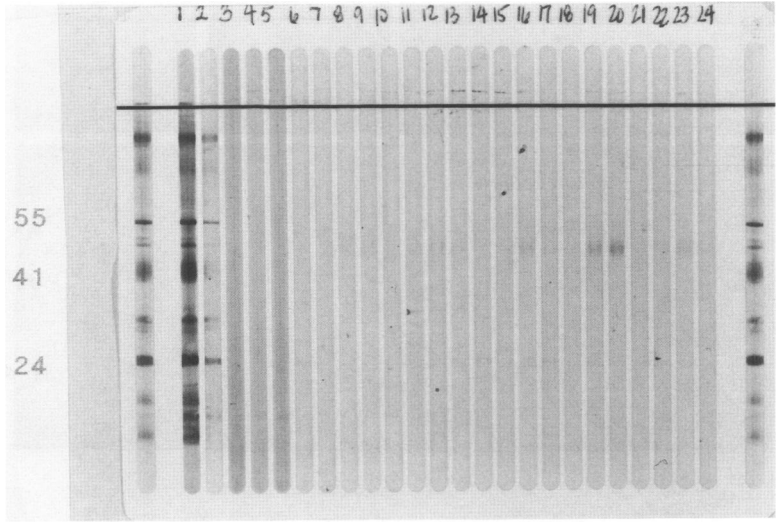

Figure 5. *Western blots of sera from HIV-infected rabbits demonstrate weak/equivocal reactivity 5 to 26 weeks after infection. Lanes 1,2: positive controls; lanes 3–5: negative control; lanes 6–13: HIV-infected rabbits—6 months; lanes 14,15: uninfected controls; lanes 16–20: HIV-infected rabbits—5 weeks; and lanes 21–24: HIV-infected rabbit—5 months. There appears to be a weakly reactive band at p51 in the sera of HIV-infected rabbits that is not present in negative controls. The significance of this reactivity is not clear because it does not correspond to a band considered critical for diagnosis in humans (p24, p31, gp41, or gp120/160) but is a band found in up to 96% of symptomatic AIDS patients (JAMA 1988;260:674).*

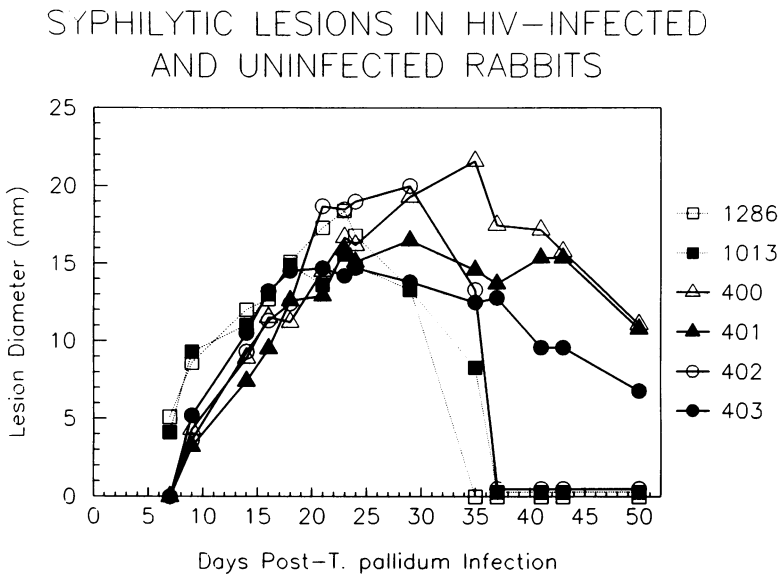

Figure 6. *Three of four HIV-infected rabbits demonstrate delayed healing of syphilitic chancres when challenged with* T. Pallidum *6 months after HIV-infection (first experiment). Each symbol represents the average diameter of eight skin lesions on the shaved skin of each rabbit. Lesions appeared about 3 days later in the HIV-infected rabbits than in the controls and did not reach the same size as the controls until 3 weeks after challenge. The lesions of three of the four HIV-infected rabbits persisted more than 2 weeks longer than those of the controls. With rechallenge with* T. pallidum *6 months after the first* T. pallidum *challenge, all six rabbits demonstrated chancre immunity (did not show cutaneous lesions). Rabbit #S 400–403 HIV-infected; rabbit #s 1013 and 1286 uninfected controls.*

tion has not been systematically reported in other animal models, including chimpanzee and rabbits.[4,37] To study the immune status of rabbits after HIV-1 infection, we superinfected four HIV-1–infected and two uninfected rabbits with *T. pallidum*, the causative agent of syphilis. Each rabbit was followed for lesion development, antibody pro-

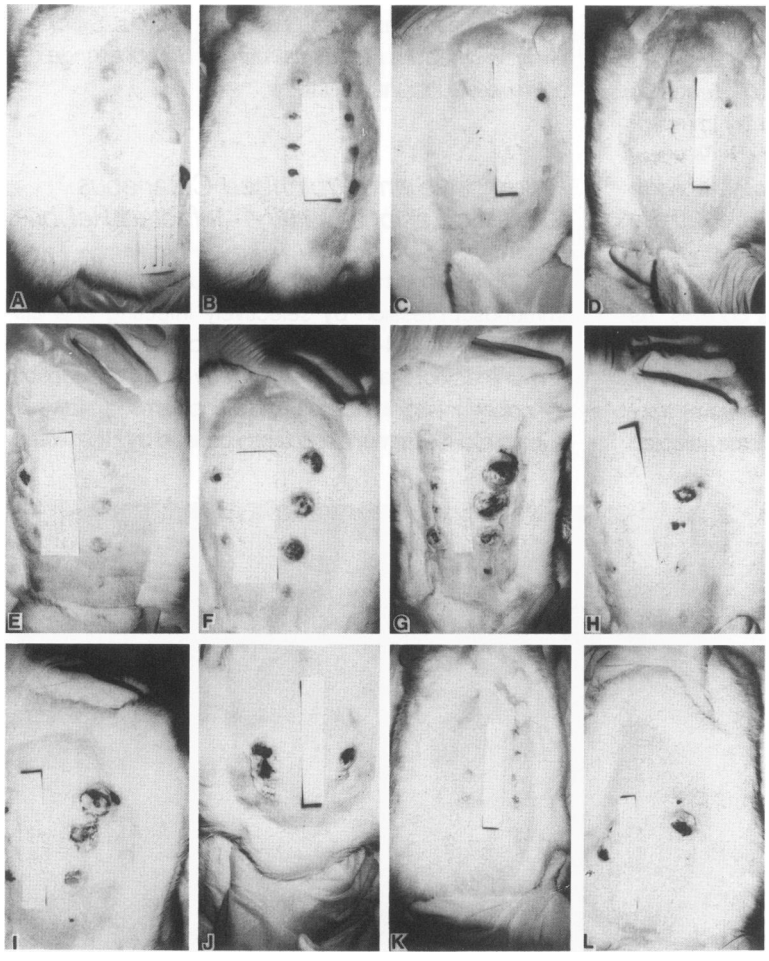

Figure 7. *Photographs of cutaneous syphilitic lesions in control and HIV-infected rabbits.* **A–D:** *Lesions of noninfected control rabbit (# 1268); days 17, 23, 39, and 52.* **E–H:** *Lesions of HIV-infected rabbit (# 400); days 17, 23, 39, and 52.* **I–K:** *Day 43 lesions of rabbits 400, 401, 402, and 403; by day 43, the lesions of the control rabbits had completely healed.*

Table 1. *The VDRL Titer of Syphilitic HIV-1–Infected and Control Rabbits*

Rabbit	HIV-1 infection	VDRL titer at day after superinfection		
		0	15	64
1013	–	–	+	+ +
1286	–	–	+	+ +
400	+	–	+	+ +
401	+	–	+	+ +
402	+	–	+ +	+
403	+	–	+	+ +

VDRL, Venereal Disease Research Laboratory.

duction for *T. pallidum* antigens, and total leukocyte counts. As shown in Figures 6 and 7, the progression of the skin lesions induced by *T. pallidum* was different between HIV-I–infected and control rabbits. The non–HIV-infected controls developed obvious gross lesions by day 7, whereas the HIV-infected rabbits had only small discolorations of the injection sites at this time. The lesions of the HIV-infected rabbits did not reach the same size as the lesions of the control rabbits until 15 to 21 days after challenge. In addition, three of four HIV-1–positive rabbits demonstrated progressive cutaneous lesions that failed to resolve by 37 days (as did the control lesions) and were not yet healed completely 8 weeks after inoculation (Figure 7). The later cutaneous lesions in the fourth HIV-1–infected rabbit had a similar time course as the controls with complete healing between 3 and 4 weeks. The delay of three of four HIV-1–infected rabbits in resolving *T. pallidum*-induced skin lesions does not appear to be due to a defect of the humoral antibody response because all of the rabbits superinfected with *T. pallidum* developed antibody to *T. pallidum* antigens similar to those of the control rabbits (Table 1). A similar result with delayed healing of syphilitic chancres was found in three of four HIV-inoculated *T. pallidum*-challenged rabbits in a second experiment (Figure 8).

Microscopic Description

Biopsies were only taken from the syphilitic chancres of one control and one HIV-infected rabbit in the first experiment on days 15, 24, and 38. Therefore any conclusions based on the histopathologic examination must be considered preliminary and will need to be amplified by more systematic studies in the future. At day 15 there was no qualitative difference between control and HIV-infected rabbits. The cutaneous *T. pallidum*-induced lesions contained heavy perivascular mononuclear cell infiltration with endothelial cell swelling, diffuse lymphocytic infiltra-

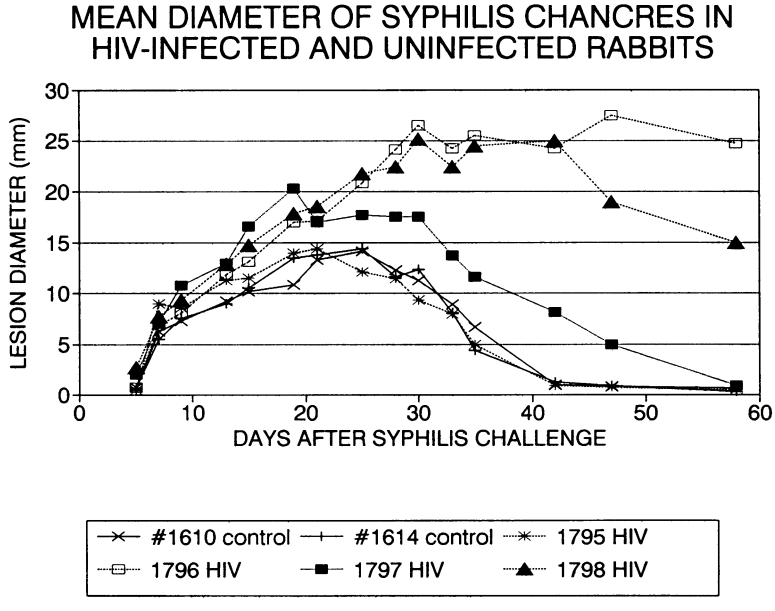

Figure 8. *Three of four rabbits demonstrate delayed healing of syphilitic chancres when challenged with* T. pallidum *4 months after HIV infection (second experiment). Each symbol represents the average diameter of eight skin lesions on the shaven back of each rabbit. The lesions of three out of four of the HIV-infected rabbits persisted after those of the control rabbits had completely healed. Measurements of the late lesions of the HIV-infected rabbits is complicated by the coalescence of individual lesions into a single large lesion. Healing of lesions in rabbits 1796 and 1798 was not completed until 5 months after* T. pallidum *challenge.*

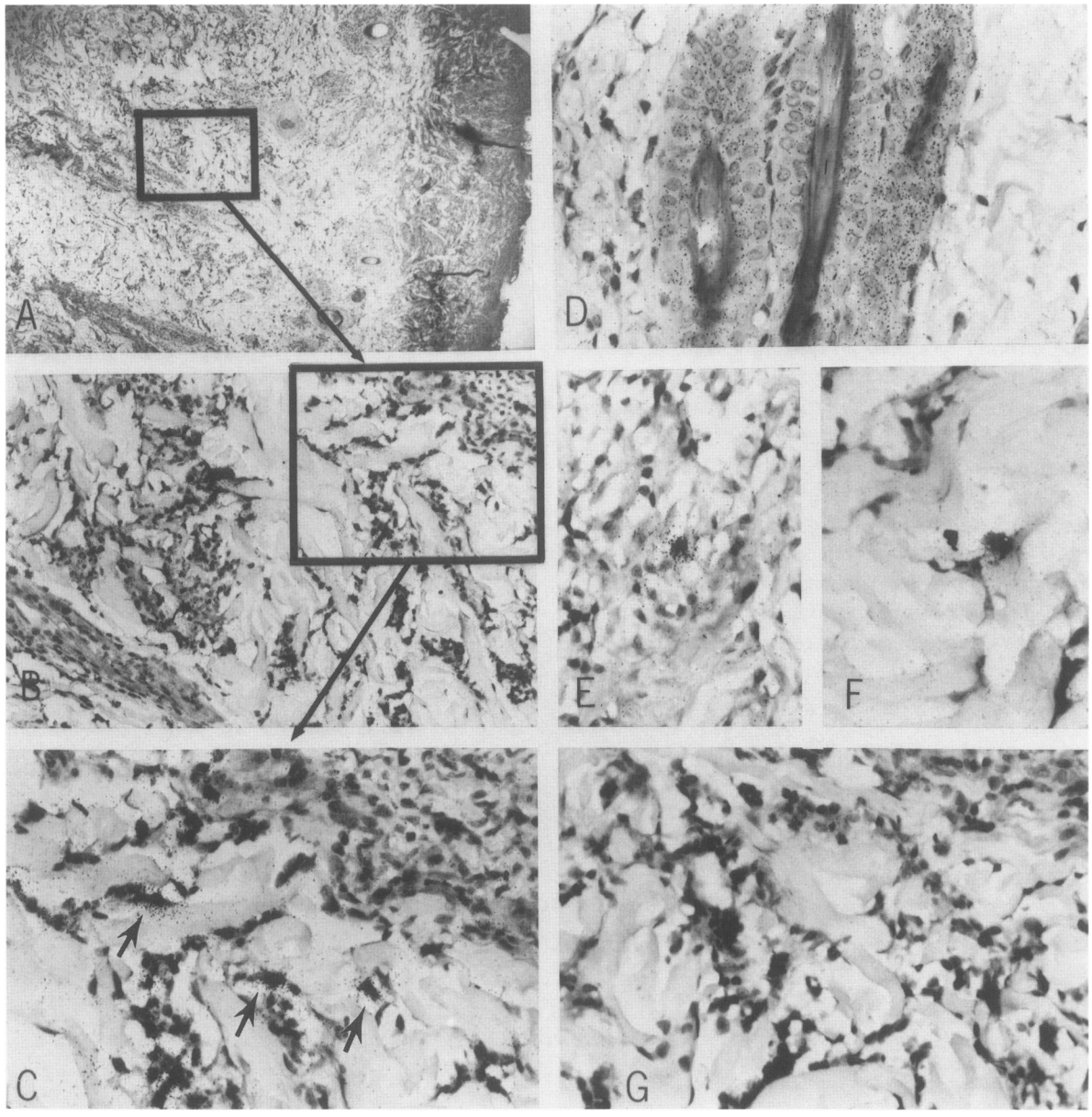

Figure 9. In situ *hybridization demonstrates HIV sequences in mononuclear cells in the dermis of HIV-infected rabbits after challenge with* T. pallidum. **A, B, and C:** *Photomicrographs of* in situ *hybridization using tat antisense probe on skin of 15-day cutaneous syphilitic chancre from rabbit 400 (HIV infected); (40×, 200×, and 400×).* **D:** *Day 15 lesion rabbit 400 using keratin probe (×400);* **E,F:** *Day 24 lesion rabbit 402 using nef antisense probe (×400);* **G:** *Day 15 lesion from rabbit 402 using nef sense probe; negative control (×400). Neither antisense nor sense probes labeled cells in skin lesions of uninfected control rabbits.*

tion and dermal edema, and extensive mononuclear infiltrate around hair follicles and in the layer of skeletal muscle underlying the dermis at the site of the lesion.[38,39] The lesions of the HIV-infected rabbits had more polymorphonuclear infiltrate and moderate leukoclasia, whereas very few polymorphonuclear cells were seen in the controls. Using the Bosma-Steiner modified silver stain,[28] many stained spiral organisms were seen extracellularly between the dermal fibers in both control and infected rabbits. At day 24, the lesion of the control rabbit (≠1013) had much less perivascular and diffuse mononuclear cell infiltration, and edema. There was early fibroblastic proliferation under the area of ulceration. The HIV-infected rabbit (≠402) still had intense perivascular mononuclear cell infiltration with increased numbers of enlarged foamy macrophages, diffuse edema, and lymphocytic infiltration with little or no necrosis. The collections of mononuclear cells resembled early noncaseating

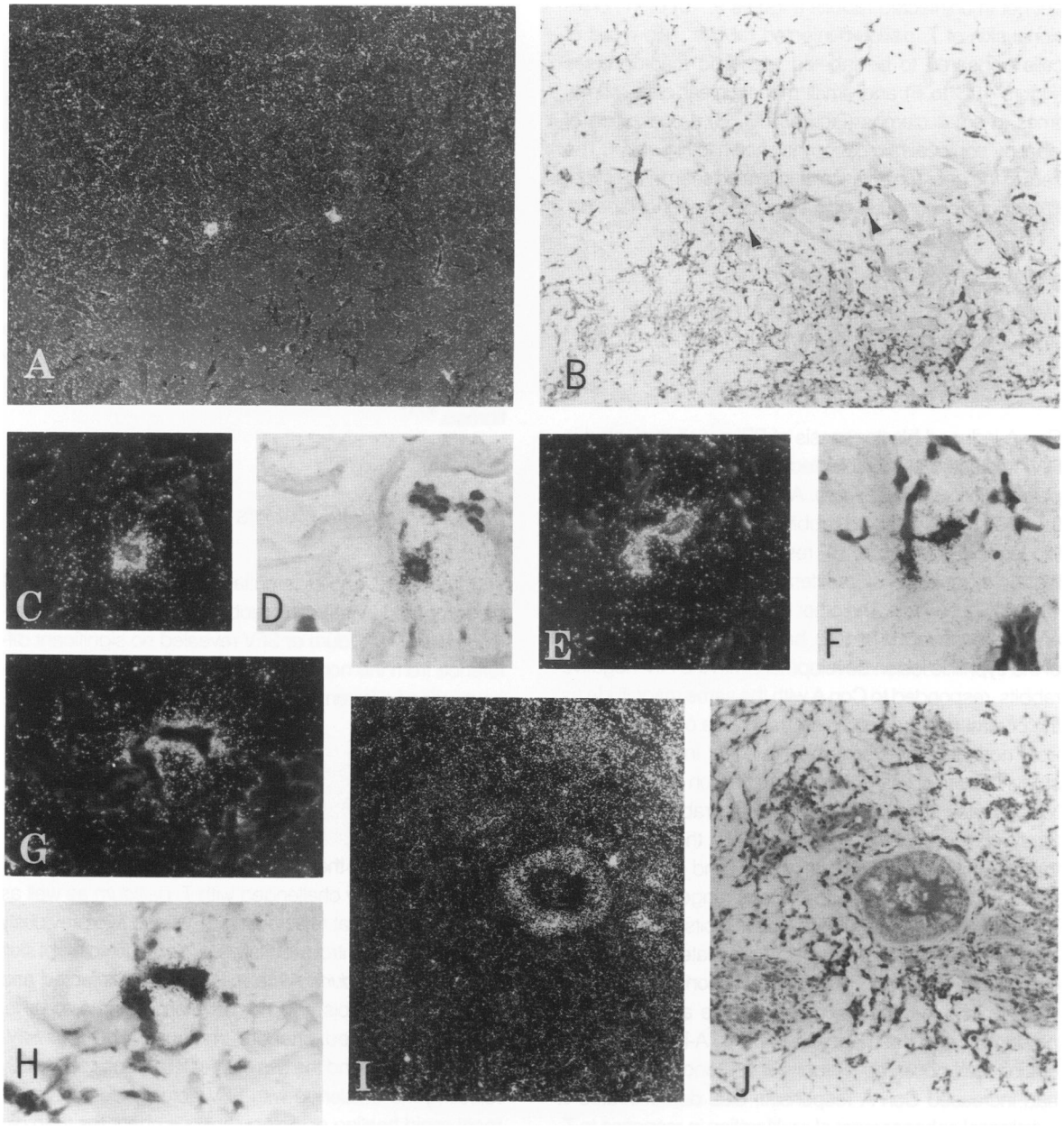

Figure 10. *Comparison of light and dark field photomicrographs of* in situ *hybridization using tat and nef probes on syphilitic skin lesions of HIV-infected rabbits.* **A,B:** *nef probe, rabbit 402, day 20 lesion (×100).* **C,D:** *nef probe, rabbit 402, day 20 lesion (×400).* **E,F:** *nef probe, rabbit 402, day 20 lesion (×400).* **G,H:** *tat probe, rabbit 400, day 15 lesion (×400).* **I,J:** *Keratin probe, rabbit 400 (positive control) (×100).*

granulomas without giant cells. At this time both sections still contained large numbers of silver-stained spiral organisms. By day 35, one control (#1013) still had demonstrable extracellular organisms, although there are many fewer than in the earlier control section. The control lesion contained tight perivascular cuffs of lymphocytes but also had sheets of plasma cells in the dermis. There was very little diffuse lymphocytic infiltrate and little, if any, dermal edema. Unfortunately the section of the HIV-infected rabbit at day 35 contained insufficient lesion for description. Although the results suggest a decreased ability of the HIV-infected rabbits to clear organisms and slower progression of the inflammatory process to healing, the histologic material is not sufficient to make definitive conclusions.

In Situ *Hybridization*

Human immune virus-specific sequences were identified in mononuclear cells in the dermis of HIV-infected rabbits

but not in uninfected rabbits (Figures 9, 10) or in nonlesional skin of *T. pallidum*-infected rabbits. Two types of cells appeared to be labeled: dendritic macrophages (Figure 10C to F) and small mononuclear cells, sometimes in small clumps (Figure 10G, H). Localization of label by confocal microscopy is shown in Figure 11. The cells in the dense perivascular infiltrates seen in the syphilitic lesions or surrounding hair follicles were not labeled.

Lymphoblastogenesis Induced by Con A and T. pallidum *Antigens in HIV-1–Infected Rabbits*

Con A-induced blastogenesis of PBLs was evaluated in HIV-1–infected and uninfected rabbits 7 weeks after superinfection with *T. pallidum.* As shown in Table 2, two of the three HIV-1–infected rabbits, with progressive syphilitic lesions (see Figure 6), responded to Con A with a higher magnitude of proliferation than those of HIV-1–negative controls. The other rabbit (#401) of this group and the fourth rabbit (#402), having a similar time course of the syphilitic lesion development with the HIV-negative rabbits, responded to Con A with the same magnitude as the control rabbits. The Con A response of lymphocytes from normal uninfected rabbits are not included in this study. We previously showed that the Con A response of lymphocytes from *T. pallidum*-infected rabbits is not different from uninfected controls.[26] Thus the response of the non–HIV-infected rabbits (#1013 and #1286) would be expected to be within the normal range. As shown in Table 3, each of the HIV-infected rabbits produced as much IL-2 as controls after being stimulated with Con A, even though the amount of IL-2 production varied among the rabbits. No correlation between the amount of IL-2 production and the magnitude of Con A-induced proliferation was observed. Rabbits #400 and #403, which had increased Con A responses, also demonstrated a substantial enhancement of proliferation in response to *T. pallidum* sonicate (Table 4).

SFV Challenge

Four of the HIV-infected rabbits and two noninfected controls were inoculated subcutaneously with an infective dose of SFV as previously described.[27] The lesions of all rabbits developed grossly in a similar manner, with rejection of the subcutaneous tumors occurring at the same time in the control and experimental rabbits. No difference was noted in the histology of rejection. Both the HIV-infected and normal controls developed the typical dermatomyofibromatous dermal lesion that showed increasing lymphocytic and polymorphonuclear infiltrate followed by necrosis of the dermal tumor surrounded by a layer of dense mononuclear cell infiltrate and healing fibrosis.[40,41]

Peripheral WBC Counts

The total WBC and differential counts of the peripheral blood of the HIV-infected rabbits before and after challenge with *T. pallidum* or SFV revealed no significant difference from the normal range of 3000 to 11,000 or percentages of different cells[42] (data not shown).

Rechallenge

Six months after the original *T. pallidum* challenge, all rabbits previously challenged with *T. pallidum* as well as the four rabbits that rejected the SFVs and two previously unchallenged control rabbits were injected into eight skin sites with *T. pallidum.* All rabbits, both HIV-infected and -uninfected controls, did not develop gross lesions, ie, they demonstrated chancre immunity. The four HIV-infected rabbits and the two uninfected rabbits previously rejecting the challenge with SFV actually demonstrated more rapid healing of their cutaneous chancres than did the control rabbits (data not shown).

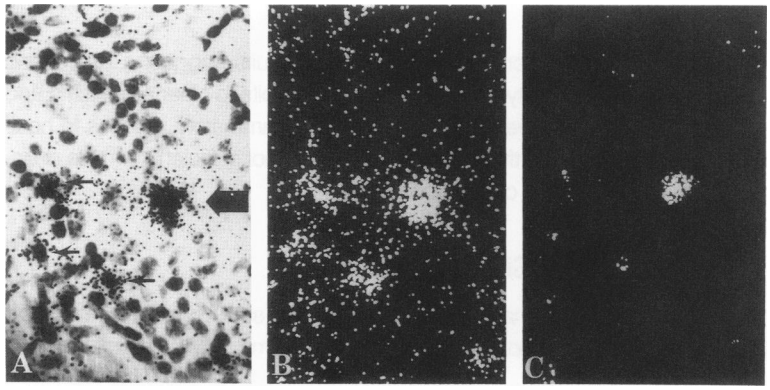

Figure 11. *Confocal microscopy of* in situ *hybridization of HIV sequences in dermis of day 28 syphilitic chancre.* **A:** *Bright field.* **B:** *Dark field.* **C:** *Confocal field. HIV sequences were detected using tat riboprobe. Rabbit 404 was infected with HIV, challenged with Shope fibroma virus 6 months later and 3 months later challenged with* T. pallidum. *Confocal examination reduces nonspecific background. A group of lymphocytes (large arrow) as well as several individual cells (small arrows) are labeled.*

Table 2. *Con A-induced Blastogenesis of Peripheral Blood Lymphocytes Derived from HIV-infected and Noninfected Rabbits*

Rabbit	HIV infection	Total IUdr uptake (CPM) at Con A concentration (μg/ml)			
		10	5	2.5	1.25
1013	–	1205	1900	1758	855
1286	–	2515	3372	2256	757
400	+	5590	9419	7679	3996
401	+	1871	1958	1319	859
402	+	1996	2224	1396	589
403	+	3232	5868	3149	1622

Unstimulated control cultures given less than 150 cpm.

Discussion

These observations demonstrate not only the establishment of a long-term latent infection of rabbits with HIV that can be activated by superinfection, but also a subtle immune deficit manifested by delayed healing of syphilitic lesions in HIV-infected rabbits. For up to 6 months after infection the HIV-inoculated rabbits remained clinically normal, did not express convincing serologic evidence of infection, and only one of eight had HIV inserts in the DNA from its peripheral blood lymphocytes. These results are somewhat different from those reported previously by Filice[17] and Kulaga et al,[18] who detected evidence of infection without superinfection or immunostimulation. However, in our experiments, HIV infection was only documented by demonstration of HIV sequences in the DNA of rabbit peripheral blood mononuclear cells after *T. pallidum* or SFV superinfection. It is unlikely that this represents survival of the injected infected H9 cells because no evidence for H9 cells was detected morphologically in peripheral blood smears and no human satellite DNA was found by PCR. It is also unlikely that this represents aborted infection, which often is observed in retroviral infections of unnatural host cells, such as avian oncoviruses in mammalian cells, because of the long time required for the presence of HIV in the PBMCs to be detectable. Thus these preliminary results indicate that HIV infection of the rabbit may produce true seronegative latency. In humans HIV may be isolated from PBMCs of homosexuals for up to 36 months before seroconversion is noted.[43] We hypothesize that latent infection may be activated by immunostimulation in the form of superinfections. This hypothesis is still only tentative because all rabbits in this study were superinfected; it is possible that evidence of infection would have surfaced without superinfection.[17–19]

We postulate further that the latent HIV infection of rabbits resides in macrophages, in particular, dendritic macrophages. This is in contrast to HTLV-1 infection in rabbits, which is easily detected in PBLs shortly after inoculation.[12–16] There is an increasing interest in the macrophage as a reservoir for HIV in humans with latent infection.[44,45] Human immune virus infection of cells other than lymphocytes was first reported in 1986 by a number of laboratories[46–49] and HIV variants that are tropic for monocytes have been isolated.[50–53] The importance of activation of T cells in production of HIV in infected cells and the role of activated macrophages (antigen-presenting cells) recently was considered.[54,55] Although HIV strain HTLV-IIIB10, which was used in this study, is tropic for human lymphocytes,[53] it also may infect rabbit monocytes. As yet we have been unable to isolate HIV using viral coculture isolation techniques as applied to clinical diagnosis.[56] However HIV sequences were detected in the dendritic macrophages in the dermis of the syphilitic lesions of the HIV-infected rabbits. Because our viral isolation procedures used mitogen-activated peripheral blood *lymphocytes* as the test sample, it seems that the one possible explanation for the failure to isolate virus is that we and others have been looking at the wrong cells. However our repeated attempts to isolate virus from the monocytes of HIV-in-

Table 3. *Con A-induced IL-2 Production of Peripheral Blood Lymphocytes (PBL) in HIV-infected and Noninfected Rabbits*

Rabbit	HIV infection	IL-2 production of PBL at Con A concentration (μg/ml)			
		10	5	2.5	1.25
1013	–	3704	3460	3232	3101
1286	–	3331	2768	2105	763
400	+	3978	3906	2805	1772
401	+	4183	3948	3423	3011
402	+	3317	2512	2191	1341
403	+	4388	4212	3366	2475

Control cell lines not supplemented with PBL culture supernates, with supernates from PBL not treated with ConA or with ConA alone give less than 50 cpm.

Table 4. In Vitro *Blastogenesis of Treponemal Antigen-specific Peripheral Blood Lymphocytes (PBL) of HIV-I–Infected and Noninfected Rabbits*

		Total IUdr uptake (cpm) at day			
		4		7	
Rabbit	HIV-I infection	−TP	+TP	−TP	+TP
1013	−	102	166 (1.6)*	104	193 (1.9)
1286	−	100	377 (3.8)	101	213 (2.1)
400	+	131	3024 (23.1)	121	6499 (53.7)
401	+	112	217 (1.9)	123	182 (1.5)
402	+	112	210 (1.9)	107	137 (1.3)
403	+	131	1228 (9.4)	130	1719 (13.2)

* Stimulation index.
TP, *Treponema pallidum.*

fected rabbits, as well as from PBLs, have been unsuccessful.

In any case, rabbits appear to develop a chronic, true latent, seronegative HIV infection, without signs or symptoms, essentially identical to postulated true latency in humans and chimpanzees infected with HIV.[4] Although there are neither clinical nor laboratory indications of infection, a subtle deficit in cellular immunity is elicited after challenge with *T. pallidum*. In one experiment, each of four HIV-infected rabbits challenged intradermally with *T. pallidum* demonstrated delayed development of the cutaneous chancre of syphilis and three of four rabbits had progressive lesions with prolonged healing as compared to non–HIV-infected control rabbits, although no differences in VDRL titers were found. In a second experiment again three of four HIV-infected rabbits had delayed healing of syphilitic chancres. We previously reported that the chancre of syphilis is a delayed-type hypersensitivity reaction (DTH). In both testicular[38] and cutaneous[39] lesions induced by inoculation of *T. pallidum* into rabbits, the early stage of the lesion features increasing T-cell infiltrate and increasing numbers of structurally intact *T. pallidum* organisms in the lesion. The organisms are located mainly extracellularly in intracellular ground substance and do not appear to be affected by the surrounding T cells. When the chancre reaches full development it has a central necrotic base surrounded by a hard indurated inflamed zone. The time required for the lesion to reach a maximum depends on the number of organisms injected; lower inoculums result in a longer time course.[57] Resolution or healing of the chancre is associated with the massive influx of macrophages with phagocytosis and digestion of the organisms.[58,59] Later lesions contain increasing numbers of plasma cells, a finding often described as typical of human chancres, most likely because the lesions are not biopsied until later in their development under clinical conditions. There is limited evidence that humoral antibody plays any role in this process and, in fact, there is considerable evidence that *T. pallidum* is not opsonized by antibody and complement in the way that other organisms are.[60] Therefore we consider the primary chancre of syphilis a manifestation of DTH that is extremely effective in clearing organisms from the infected site.

Differences between the HIV-infected and noninfected rabbits in the evolution of the syphilitic chancre suggest a deficit in T-cell or macrophage function. Because the early development of the lesion depends on the infiltration of T cells, the later appearance of the lesions in HIV-infected rabbits suggests a deficiency in the number of T cells or the ability of T cells to identify the foreign organisms and localize at the site of infection. It is unlikely that the enlarged prolonged lesions could be due to such a mechanism as the later lesions contain large numbers of T cells. However it is possible that the earlier ineffective response allows more *T. pallidum* to be produced, thus leading to larger numbers of organisms and a more extensive and prolonged lesion because the DTH mechanism requires more time to bring the lesion under control. Alternatively there could be a defect in the ability of T cells to produce inflammatory cytokines or in the number and/or function of macrophages. Thus, although there may be large numbers of T cells or macrophages in the later lesions, they may be relatively ineffective in clearing the infecting organisms. Preliminary measurements of blood counts, IL-2 production, and mitogenic responses do not support a T-cell abnormality. On the other hand, a selective T-cell deficit cannot be ruled out. The ability of HIV-infected rabbits to reject SFV lesions normally suggests that rejection of SFV is somehow different from clearance of *T. pallidum* from chancres. Previous studies indicated cytotoxic T cells were active in SFV rejection,[61] whereas a classic DTH reaction requiring macrophages is active in syphilis lesions. Further studies on the role of T cells and macrophages in the protracted syphilitic lesions in HIV-infected rabbits are required to determine the nature of the immune deficit in this model.

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