Low Levels of HIV-1 Infection in Cutaneous Dendritic Cells Promote Extensive Viral Replication upon Binding to Memory CD4+ T Cells

By Melissa Pope,* Stuart Gezelter,* Nancy Gallo,‡ Lloyd Hoffman,§ and Ralph M. Steinman*

From the *Laboratory of Cellular Physiology and Immunology, The Rockefeller University, New York 10021; the ‡New York Firefighter's Skin Bank, New York Hospital, New York 10021; and the §Division of Plastic Surgery, Cornell University Medical College, New York 10021

Summary

Earlier work has identified a cell population that replicates HIV-1 in the absence of standard T cell stimuli. The system consists of dendritic cells and memory T lymphocytes that emigrate from organ cultures of human skin and together support a productive infection with HIV-1. These emigrants resemble cells that can be found in mucous membranes and that normally traffic in afferent lymph. Here, we report that a low level of infection in the dendritic cell can initiate extensive HIV-1 replication in cocultures with T cells. First we extended our earlier work to larger skin specimens from cadavers. As long as the organ cultures were set up within 36 h of death, the emigrant leukocytes were comparable to cells from fresh surgical specimens in number, phenotype, and function. These mixtures of dendritic cells and T cells provided the milieu for a productive infection with several virus isolates. When purified dendritic cells were separately pulsed with virus and then mixed with T cells that had not been pulsed with HIV-1, active infection ensued. The infectivity of HIV-pulsed dendritic cells persisted for at least 1.5 d in culture, but was blocked if AZT was added during that time to block reverse transcription in the dendritic cells. The number of copies of proviral DNA in the dendritic cells corresponded to <100 copies per 5 × 10⁴ cells, but upon mixing with T cells, >10⁴ copies were found 5–7 d later. By contacting syngeneic T cells, extralymphoid depots of dendritic cells—even with a low viral burden as has been reported in vivo—may contribute to chronic HIV-1 replication in infected individuals.

The mucosal surfaces of several organs that are exposed to HIV-1 during sexual transmission are similar to skin, although lacking the outer cornified layer. Both skin and mucosal surfaces consist of a stratified squamous epithelium in which dendritic cells are positioned above the basal layer (1–3). Cutaneous dendritic cells also express the HIV-1 receptor CD4 (4, 5). HIV-1 has been detected in scattered cells in rectal (6) and cervical (7) biopsies from infected humans, and SIV-1 has been found in the cervical and vaginal mucosa of infected macaques (8, 9). To characterize events that might take place during sexual transmission of HIV-1, particularly involving the role of CD4+ dendritic cells in viral replication, we chose to study skin because it is more generally available than mucous membranes (especially if cadaver donors can be used).

When explants of surgically excised skin were placed in organ culture, both dendritic cells and T lymphocytes emigrated into the medium (10, 11). The emigrated T cells were TCR-α/β+, either CD4+ or CD8+, and they expressed a "memory" phenotype (i.e., they were rich in

CD45RO and LFA-3 but weak in activation antigens [CD25, CD80, MHC class II]) (10). Some of the T cells formed tight conjugates with some of the dendritic cells, but the lymphocytes remained small and were not in cell cycle. Nevertheless, when HIV-1 was added, active infection took place (12). Infection with HIV-1IIIB required the joint presence of both dendritic cells and T cells, but no additional stimuli such as FCS, IL-2, or mitogens.

In this study, we demonstrated that similar events would take place with cells derived from cadaver skin donors. Then we asked if the dendritic cell could be infected and, if so, if the infection could be transmitted to syngeneic T cells. We show that when dendritic cells are pulsed with HIV-1, cultured for up to 1.5 d, and then mixed with T cells that have not been exposed to virus, productive infection ensues. The infectivity of virus-pulsed dendritic cells is fully blocked by AZT, as long as this reverse transcriptase inhibitor is included for 1.5 d before adding T cells. The level of full-length reverse transcripts in dendritic cells is low, <100 copies per 5 × 10⁴ cells. Because these two cell

types are known to interact continuously in situ, especially in afferent lymph and some mucosal surfaces, the data suggest a role for small numbers of infected dendritic cells in nonlymphoid tissues during the chronic replication and transmission of HIV-1.

Materials and Methods

Culture Medium

RPMI 1640 (Cellgro; Fisher Scientific Co., Pittsburgh, PA) was supplemented with 50 μM 2-ME (Sigma Chemical Co., St. Louis, MO), 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco BRL, Gaithersburg, MD), 10 mM Hepes (Gibco BRL), 2 mM L-glutamine (Gibco BRL), and 10% FCS (Gibco BRL) or 10% pooled normal human serum (NHS;[1] C-Six Diagnostics, Inc., Mequon, WI). For convenience, FCS medium was used for skin organ culture and either FCS- or NHS-containing medium for subsequent infection and cell culture.

Preparation of Cell Suspensions

Split thickness skin was harvested from individuals undergoing elective plastic surgery or from cadavers within 24 h of death. The surgical skin would otherwise have been discarded and was used with informed consent. Cadaver skin that was authorized for use in research was obtained from the New York Firefighter's Skin Bank. A maximum of three 3 × 3-cm pieces of skin were placed into organ culture in 15 ml of medium in 10-cm tissue culture dishes, (model 3003; Falcon Plastics, Cockeysville, MD) for 2–4 d, and the migrated cells were harvested (10). The cells often were sorted into dendritic cells, T cells, and dendritic–T cell conjugates using a FACStar^PLUS® (Becton Dickinson Immunocytometry Systems, Inc., Mountain View, CA) (10). Small (free T cells) and large (dendritic cells and dendritic cell–T cell conjugate) cells were separated on the basis of forward and side light scatter. The large cell fraction was then divided into CD3+ dendritic cell–T cell conjugates and CD3− free dendritic cells. In most experiments, we studied the function of sorted dendritic cells, T cells, or mixtures, so none of the cells were detectably stained with mAbs; however, the anti-CD3 mAb that was used to prepare dendritic cell–T cell conjugates (above) did not enhance HIV-1 infection. Under the hemocytometer, purified dendritic cells and T cells typically contained <2% contaminating conjugates, and often no conjugates were seen in 300 cells. Nevertheless, to ensure removal of any contaminating conjugates, we treated the dendritic cells with anti-CD2 (anti–T cell) and T cells with anti–HLA-DR (anti–dendritic cell) coated magnetic Dynabeads (DYNAL, Inc., Great Neck, NY). Anti-CD19–coated Dynabeads were included as a control. Following the manufacturer's guidelines, beads were added for 30 min with rotation in the cold (five beads per target cell). Bead-bound cells were removed using a magnetic particle concentrator (DYNAL), and the unbound cells were collected. In some experiments, an aliquot of the T cells was stained with CD4/CD8 simultest mAbs (Becton Dickinson Immunocytometry Systems) to prepare sorted CD4+ and CD8+ fractions using the FACStar^PLUS® (Becton Dickinson Immunocytometry Systems) with laser excitation of 200 mW at 488 nm (Innova 90-5 Argon laser; Coherent, Inc., Palo Alto, CA). Control populations were peripheral blood mononuclear cells (PBMC), resting T cells, and Staphylococcal enterotoxin E (SEE; Toxin Technology, Inc., Sarasota, FL) superantigen-activated T blasts (12).

Light Microscopy of Cytospin Smears

Cell cytospins were stained with primary mAbs and 3 μg/ml horseradish peroxidase (HRP) F(ab')₂ donkey–anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) (10). After four washes, HRP substrate (Stable DAB; Research Genetics, Huntsville, AL) was added for 3 min, replaced with fresh DAB for another 3 min, and washed with distilled water. Cytospins were coverslipped and photographed on a Nikon Optiphot Microscope (Morell Instrument Co. Inc., Melville, NY). mAbs included anti-CD3 (Leu 4), anti-CD4 (Leu 3a), anti-CD8 (Leu 2), anti-CD62L (Leu 8), anti-CD69, anti–CD80/B7-1, and anti–CD86/B7-2 (BDIS); anti–HLA-DR (9.3C9, HB180) and anti–CD58/LFA-3 (HB205) from American Type Culture Collection (Rockville, MD); anti–Ki-67 (MIB-1; AMAC, Inc., Westbrook, ME); anti-HIV p24 (hybridoma 183; clone H12-5C [13]; AIDS Research and Reagent Program); anti-CD25 (AM47) from Dr. S. Fu (University of Virginia, Charlottesville, VA); anti-CD45RA (4G10); and anti-CD45RO (UCHL-1) from Dr. P. Beverly (University College London Medical School, London, UK).

FACS® Analyses

Emigrated skin cells were double immunostained as previously described (10). PE-anti–HLA-DR was used to identify strongly positive dendritic cells and a panel of mAbs with FITC–anti-Ig was used to phenotype the T cells and dendritic cells.

T Cell Proliferative Responses

To detect T cell proliferation in the emigrant skin cells, 4 × 10⁴ cells were cultured in triplicate 96-well round-bottomed trays (Flow/ICN, Horsham, PA) without or with 10% human rIL-2 (Boehringer Mannheim, Indianapolis, IN), 2 ng/ml SEE, or 3 μg/ml Con A (Boehringer Mannheim). After 3 d, proliferation was monitored either by [³H]thymidine ([³H]TdR) uptake or by staining cell smears (cytospins) for the Ki-67 nuclear antigen expressed in cycling cells (14). The mixed leukocyte reaction stimulating activity of a range of skin emigrant doses was assessed with 1.5 × 10⁵ allogeneic T cells (15).

Virus Isolates

Patient-derived HIV-1 isolates were kindly donated by Dr. Ruth Connor (Aaron Diamond AIDS Research Center, New York, NY [16]). The isolates had been characterized as syncytia inducing (SI) or nonsyncytia inducing (NSI) using MT-2 cells (17). B 5/85 and B 11/88 are NSI and SI isolates from the same individual at different times after infection, C 5/84 and C 2/86 are NSI and SI isolates similarly isolated from another patient, and D 3/86 is an NSI isolate obtained from a third patient. Virus-containing supernatants were prepared in SEE-activated PBMC or the CEM T cell line (12). Viral supernatants were treated with RNase-free DNase I (Boehringer Mannheim) at 50 U/ml for 30 min at 37°C.

HIV-1 Infection of Test Cell Suspensions

10⁵ viable cells were cultured for up to 9 d in 200 μl in 96-well round-bottomed trays (Flow/ICN). When mixtures of

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[1]Abbreviations used in this paper: AZT, 3′-azido-3′-deoxythymidine; MOI, multiplicity of infection; NSI, nonsyncytia inducing; PBMC, peripheral blood mononuclear cell; RTase, reverse transcriptase; SI, syncytia inducing; SEE, staphylococcal enterotoxin.

sorted dendritic cells and T cells were studied, 3 × 10⁴ dendritic cells and 6 × 10⁴ T cells were added per well. Live virus was added for 1.5 h at 37°C (at a multiplicity of infection [MOI] of 0.05–0.1). The cells were washed two to four times (12), and fresh medium was added. When the dendritic cells or T cells were separately pulsed with virus, the cells were washed four times (to reduce carryover of free virus) and mixed with their non–HIV-pulsed counterpart immediately or >12 h later. To show that virus-pulsed dendritic cells were infected, the cells were treated with 10 μM 3′-azido-3′-deoxythymidine (AZT; Sigma Chemical Co.) before (30 min) and during (90 min) the exposure to HIV-1, and AZT was added back for further culture after exposure to virus. In some experiments, 5 × 10⁴ dendritic cells or T cells were placed into Eppendorf tubes (Sarstedt, Inc., Newton, NC) and pulsed with HIV-1 with or without AZT (as described above). The cells were then washed six times in 500-μl vol of medium, centrifuged for 2–3 min at 2,500 rpm (using a Microspin 12S; Sorvall Instruments, DuPont), and counted. The HIV-pulsed dendritic cells were mixed with non–HIV-pulsed T cells (and vice versa) immediately or >12 h later (3 × 10⁴ dendritic cells with 6 × 10⁴ T cells). The >12 h cultures were carried out in 96-well round-bottomed trays, in the presence or absence of 10 μM AZT. Before mixing with non–HIV-pulsed cells, the cultured HIV-pulsed cells (with or without AZT) were transferred to tubes, washed as described above, and counted. To rule out carryover of AZT into the subsequent culture, we ran controls by adding dendritic cells that had been exposed to AZT (or not) in the absence of virus to HIV-pulsed dendritic cell–T cell cocultures.

Detection of Infection with HIV-1

PCR. Standard proteinase K digestion (Boehringer Mannheim) was used to prepare DNA 16–108 h after pulse with live virus (12). 25-μl aliquots with 5 × 10⁴ cell equivalents were stored at −20°C ready for amplification by PCR. Both HIV-1 *gag* sequences (primers SK38 and SK39 [18]) and HLA-DQ sequences (primer pairs GH26 and GH27 [19]) were amplified in the same tube (12). PCR products were resolved on an 8% polyacrylamide gel run in 0.5 × TBE. Gels were dried and exposed to XAR-5 film (Eastman Kodak Co., Rochester, NY). The number of virus copies was estimated by comparison with the cell line ACH-2, which contains one copy of provirus per cell (20). A standard curve (see Figs. 3 and 4, *Controls*), was set up and labeled: 4 = 10⁴, 3 = 10³, 2 = 10², 1 = 10¹, 0 = 1, and − = 0 ACH-2. 10⁵ HIV-1 negative fillers were included to provide a constant HLA-DQ signal.

Reverse Transcriptase [RTase] Assay. At different times after exposure to virus, triplicate 10-μl aliquots of culture supernatants were harvested into separate wells of a 96-well V-bottomed tray (Flow/ICN) and stored at −20°C. RTase activity in the supernatants was detected using a micro RTase assay (12).

Detection of HIV-1 p24 Antigen. The 183 mAb was used to detect p24 antigen at the single-cell level by staining cytospins (see above). The entire cytospin (corresponding to 25% of the microculture) was then scanned for stained syncytia, which were the principal location of newly synthesized p24, as described (12).

Results

An earlier study showed that mixtures of cutaneous dendritic cells and T cells were permissive to infection with HIV-1 (12). We reasoned that this cellular system might model events at mucosal surfaces during acute infection with HIV-1. We first extended the culture system to cadaver skin so that larger numbers of cells were available. Then we assessed the relative roles of dendritic cells and memory CD4⁺ T cells in the initiation of a productive and cytopathic infection with different HIV-1 isolates. We found that a low level of infection underlies the capacity of
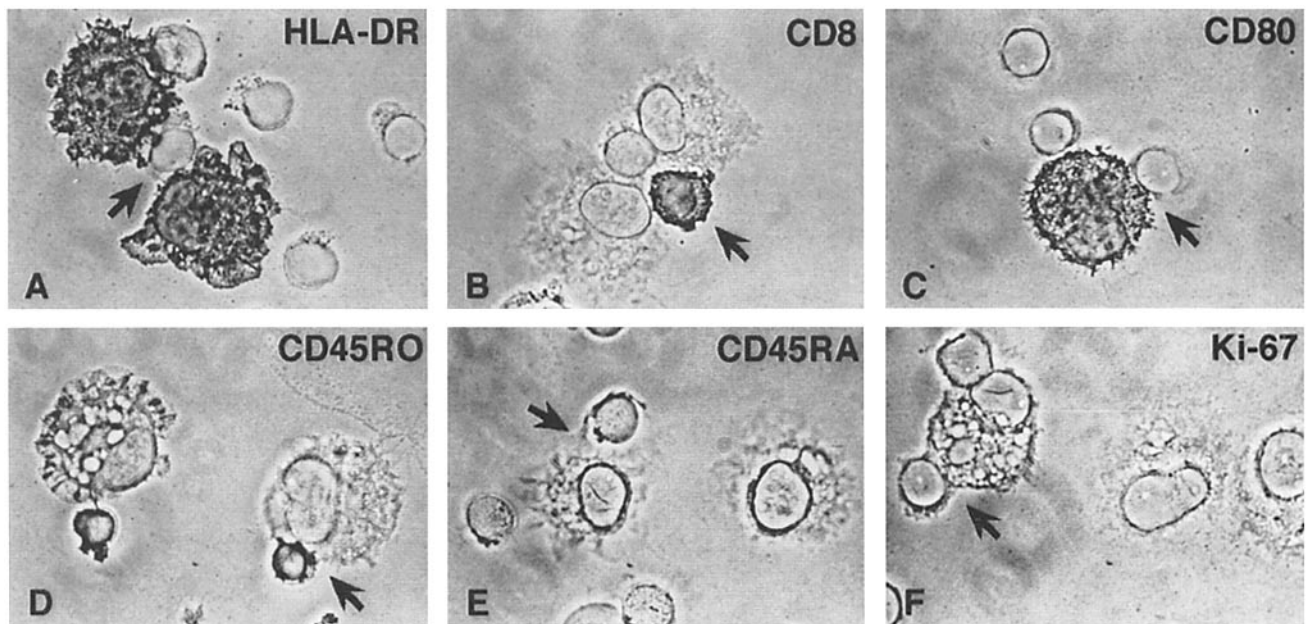

Figure 1. Cadaver-derived emigrants contain memory T cells and dendritic cells. Cadaver skin was placed into culture and the cells were allowed to migrate during the next 4 d. Cytospins were prepared and immunoperoxidase stained for the expression of HLA-DR, CD8, CD80, CD45RO, CD45RA, or Ki-67 (*A–F*). In each frame, an arrow marks a small T cell conjugated to a large dendritic cell. ×500.
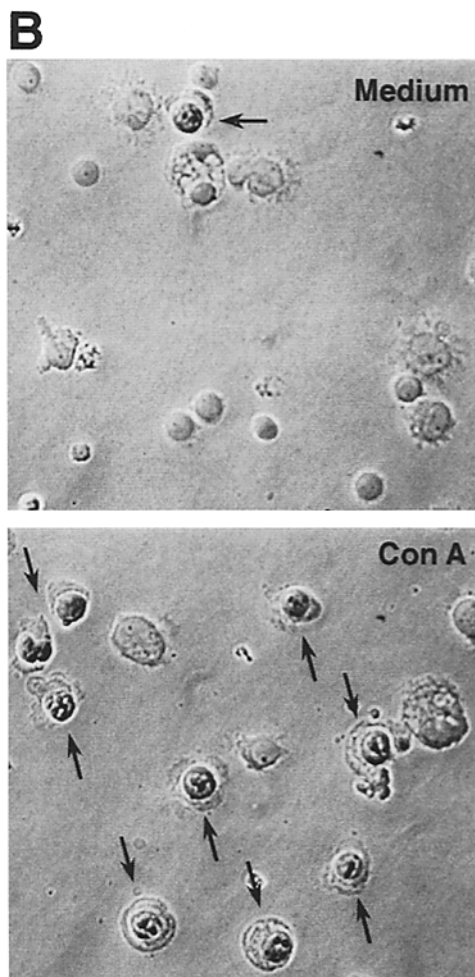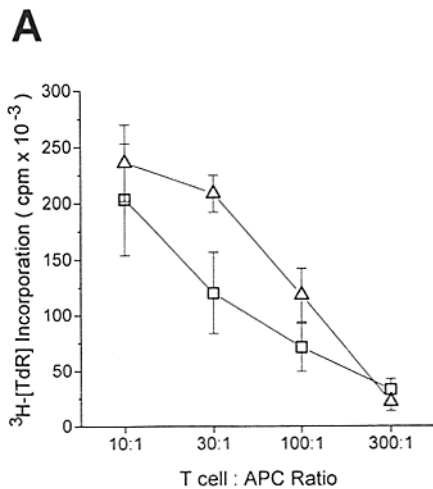
A

B

Figure 2. Dendritic cells and T cells migrating from cadaver-derived skin are functional. (*A*) Graded doses of irradiated skin cell emigrants (APC) were cultured for 6 d with 1.5×10^5 T cells from another individual. The MLR was monitored by [^{3}H]TdR incorporation during the last 8 h of culture. Representative data (mean cpm ± SEM of triplicate cultures from three similar experiments) illustrate the similar MLR stimulatory capacities of skin cells from surgical (live donor, —□—) and cadaver (—△—) specimens. Control cultures of the highest dose of stimulator or responder cells alone exhibited minimal [^{3}H]TdR uptake: cadaver emigrants, 1,564 ± 243; surgical emigrants, 1,244 ± 245; T cells, 725 ± 86.

cutaneous dendritic cells to replicate HIV-1 upon contact with syngeneic memory T cells.

Cadaver Skin Is Similar to Surgical Skin as a Source of Dendritic Cells and T cells. Preliminary experiments revealed that viable cells could migrate from the skin if they are cultured up to 72 h after death. Functional capacity was significantly reduced, however, if the cultures were begun >36 h after death (MLR stimulation, proliferation to mitogens; data not shown). We restricted our work to cadaver specimens that were obtained <36 h after death. An average of 52,000 ± 5,300 leukocytes emigrated per square centimeter of skin (mean ± SEM from the first 11 experiments). This is comparable to the yields from surgical specimens (57,000 ± 6,200/cm²).

To examine the phenotype of the emigrated cells, particularly the activation state of the T cells that had bound to the dendritic cells (Fig. 1 *A*, *arrows*), we immunolabeled cytospins. To illustrate the results from a large number of antibodies, some of the results are shown (Fig. 1). The cadaver skin emigrants contained two populations: large, irregularly shaped, HLA-DR$^+$ CD3$-$ dendritic cells (Fig. 1 *A*) and small- to medium-sized CD3$^+$ HLA-DR$^-$ T cells. CD1a expression by the dendritic cells was heterogeneous, ranging from undetectable to strong. The dendritic cells stained for B7-1/CD80 (Fig. 1 *C*), B7-2/CD86, and IL-2 receptor/CD25. Both dendritic and T cells stained for LFA-3/CD58. Most T cells stained for CD45RO, and <5% had detectable CD45RA (Fig. 1, *D* and *E*), whereas the dendritic cells exhibited variable (undetectable to moderate) CD45RO labeling (Fig. 1 *D*). None of the emigrated cells expressed the Ki-67 antigen of cycling cells (Fig. 1 *F*) (14), or the CD62L or L-selectin homing receptor.

FACS® analyses were also carried out using PE-labeled anti–HLA-DR (to distinguish dendritic cells from T cells) plus a panel of mAbs and an FITC label to phenotype the two cell types. The results were identical to those reported from migrants from live donor skin (10). In brief, the dendritic cells expressed high levels of class I and II MHC products, as well as many costimulatory and adhesion molecules (CD11a and CD11c, CD29, CD54, CD58, and CD80). Both CD4$^+$ and CD8$^+$ T cells were present, and these expressed the memory phenotype CD45RA$^{-/weak}$, CD45RO$^+$, and LFA-3$^+$. The T cells had minimal levels of the activation markers HLA-DR, CD25, and CD80.

The funcitonal capacity of the dendritic cells in the cadaver skin cell emigrants was assessed by their ability to stimulate proliferation of T cells in the allogeneic MLR. A fixed number of T cells were cultured with a range of doses of irradiated skin cell emigrants (from live and cadaver donors). Fig. 2 *A* shows data from one of three representative

(*B*) Cytospins were prepared from emigrants cultured for 3 d with either Con A, SEE, IL-2, or medium. Cell activation was assessed by staining with an mAb specific for the Ki-67 nuclear antigen in cells cultured with medium (*top*) or Con A (*bottom*). Ki-67$^+$ lymphocytes (*arrows*) are rare when cells are cultured in medium without stimulant, but they are abundant with a stimulant. ×300.

Table 1. *Proliferation of Cadaver-derived Cutaneous T Cells*

Stimulus	[³H]TdR incorporation	Percent Ki-67⁺
None	1836 ± 208	1.8 ± 0.4
IL-2	29,849 ± 1117	14.1 ± 1.9
SEE	50,936 ± 1776	35.2 ± 2.1
Con A	119,238 ± 6057	60.3 ± 3.0

Triplicate samples of 4×10^4 skin cell emigrants were cultured in medium alone (*None*) or with 3 μg/ml Con A, 2 ng/ml SEE, or 10% IL-2. DNA synthesis was measured by the amount of [³H]TdR incorporated in the final 8 h of the 3-d cultures. Results from one of three similar experiments are provided [mean cpm ± SEM]. Cycling T cells were also identified by Ki-67 staining. At least five fields of 200 cells were counted and the results were expressed as the percentage of Ki-67⁺ cells (mean ± SEM).
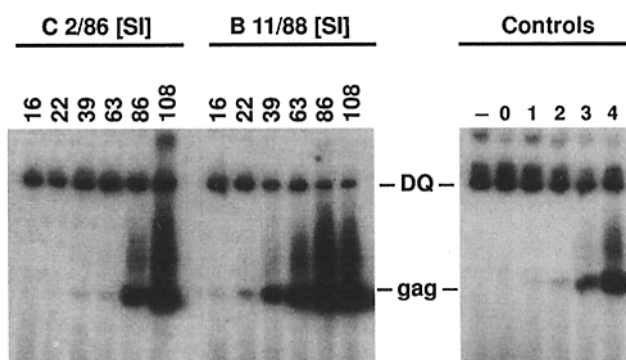

Figure 3. Primary HIV isolates replicate in skin cell emigrants obtained from cadaver donors. Cutaneous emigrants were pulsed with 0.05–0.1 MOI of the SI isolates B 11/88 or C 2/86 and cultured for up to 5 d. DNA was extracted from cells at 16, 22, 39, 63, 86, and 108 h after exposure to the virus. HIV *gag* (*gag*) and HLA-DQ (*DQ*) DNA sequences were amplified by PCR from each sample. Amplified products from one of two similar experiments are presented. The controls in this and other figures are graded doses of ACH-2 cells ($0 = -$, $10^0 = 0$, $10^1 = 1$, $10^2 = 2$, $10^3 = 3$, $10^4 = 4$) mixed with 10^5 copies of HLA-DQ.

experiments. Irradiated cells from live or cadaver donors were potent stimulators for T cell proliferative responses.

Cadaver-derived skin cell emigrants were also cultured with several T cell stimuli: SEE, Con A, and IL-2. After 3 d of culture, proliferative responses were measured using two methods, [³H]TdR uptake and expression of the Ki-67 cell cycle antigen. Each stimulus induced strong responses (Fig. 2 *B* and Table 1) comparable to results with cells from live donor skin cell emigrants (10). In the absence of stimulus, <2% of the cells were in cell cycle. We conclude that the cells that emigrate from cadaver skin are immunocompetent mixtures of dendritic cells and nonactivated memory T cells.

Cadaver-derived Skin Cell Emigrants Support Replication of HIV-1. Cadaver-derived skin cells supported HIV-1 replication in a manner similar to that of cells from live donor specimens. Our protocol was to add HIV-1 for 90 min at 0.05–0.1 MOI, wash, and culture for 16–108 h. The extent of infection was determined by measuring the level of proviral DNA by PCR amplification of HIV-1 *gag*-specific DNA sequences. For two SI isolates of HIV-1, virus burden increased from 100 to $\geq 10^4$ copies per 5×10^4 cells within 4–5 d (Fig. 3). The kinetics of infection with the NSI isolates was slower than that with SI isolates (10–100 copies per 5×10^4 cells after 4 d of culture; not shown, but see below), but at later time points high virus copy numbers were observed. To ensure that the infection was not being stimulated with mitogen or IL-2 that could have been carried over from the cultures used to prepare virus, we studied two isolates that had been separately grown in CEM cells (without SEE or IL-2) and in SEE-induced T blasts and IL-2. In a separate experiment (not shown), infection kinetics similar to those in Fig. 3 were observed when the B 11/88 SI isolate was prepared in either T blasts or CEM cells. Therefore, several isolates of HIV-1 replicate vigorously in mixtures of dendritic cells and T cells, but exogenous stimuli appear to not be required.

Comparison of Infection of Sorted Dendritic Cells and T Cells, and Their Mixtures. In previous studies, we showed that dendritic cell–T cell conjugates, but not purified dendritic cells or T cells, were productively infected by the long-standing lab isolate of HIV-1, III$_B$ (12). The infectivity of sorted dendritic cells, T cells, and dendritic cell–T cell conjugates was tested with more recently derived NSI and SI isolates. Each cell fraction was exposed to virus, washed, and cultured for 36 h. HIV-1 *gag* sequences were amplified by PCR and virus levels assessed in each cell fraction relative to two types of controls: resting T cells or SEE-induced T blasts (Fig. 4, *rT, TB*). When cells were exposed to either NSI (B 5/85; Fig. 4 *A*) or SI (B 11/88; Fig. 4 *B*) HIV-1, the conjugate fraction exhibited the strongest HIV-1 DNA signal 36 h after adding virus (>100 and >1,000 copies per 5×10^4 cells for NSI and SI isolates, respectively). The level of infection in mixtures of cutaneous leukocytes was often comparable to that seen with activated T blasts. Free dendritic cells and T cells, however, had only <100 copies per 5×10^4 cells (Fig. 4, *A–C*).

To prove that the PCR signals were caused by infection rather than by carryover of DNA in the virus preparation (21), replicate cultures were set up in which AZT was included to block reverse transcription. AZT markedly blocked infection with both NSI and SI isolates (Fig. 4, *A* and *B*). The infection in the dendritic cell fraction was also sensitive to AZT (Fig. 4 *C*; compare "−" and "+"), indicating that reverse transcription can be completed in this population.

Productive Infection with HIV-1 Requires CD4⁺ but Not CD8⁺ T Cells. Productive infection was monitored by measuring the release of RTase into the medium. With all five viral isolates, including the Ba-L monocytotropic isolate (22), sorted dendritic cells and sorted T cells did not support a productive infection, but their mixtures were comparable to bulk unsorted populations (Fig. 5). Given that both CD4⁺ and CD8⁺ T cells could conjugate to den-
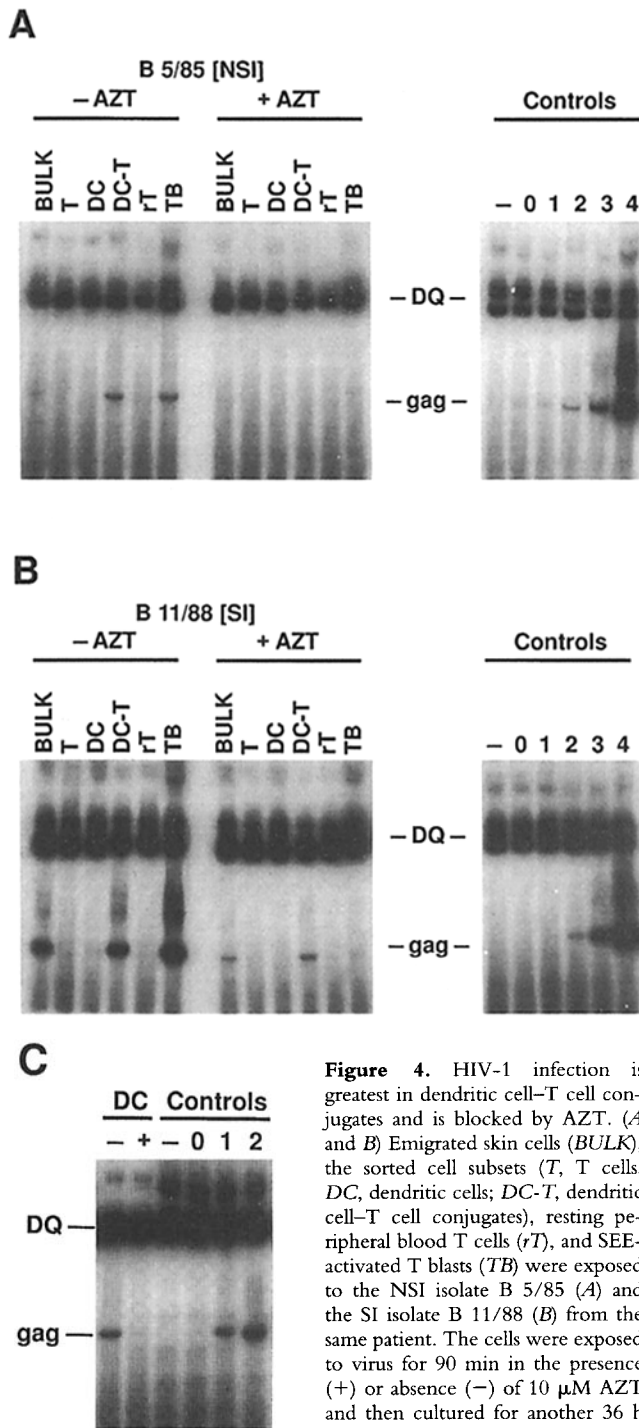
A

B 5/85 [NSI]

−AZT　　+AZT　　Controls

BULK T DC DC-T rT TB　　BULK T DC DC-T rT TB　　− 0 1 2 3 4

— DQ —

— gag —

B

B 11/88 [SI]

−AZT　　+AZT　　Controls

BULK T DC DC-T rT TB　　BULK T DC DC-T rT TB　　− 0 1 2 3 4

— DQ —

— gag —

C

DC　Controls

− +　− 0 1 2

DQ —

gag —

Figure 4. HIV-1 infection is greatest in dendritic cell–T cell conjugates and is blocked by AZT. (A and B) Emigrated skin cells (BULK), the sorted cell subsets (T, T cells; DC, dendritic cells; DC-T, dendritic cell–T cell conjugates), resting peripheral blood T cells (rT), and SEE-activated T blasts (TB) were exposed to the NSI isolate B 5/85 (A) and the SI isolate B 11/88 (B) from the same patient. The cells were exposed to virus for 90 min in the presence (+) or absence (−) of 10 μM AZT and then cultured for another 36 h before preparing the lysates and amplifying HIV gag and HLA-DQ DNA sequences. Resolved PCR products are representative of three similar observations. (C) Sorted dendritic cells were depleted of contaminating conjugates using anti-CD2-coated magnetic beads and exposed to the B 11/88 isolate in the presence (+) or absence (−) of AZT. 36 h later, the DNA was prepared and HIV-gag (gag) and HLA-DQ (DQ) DNA amplified. Similar reductions in the HIV-gag signals by AZT treatment (from 100 to <10 gag copies) of either dendritic cells or T cells (not shown) have been observed in six separate experiments.

dritic cells, it was possible that either could signal dendritic cells to become permissive to HIV-1. When the mixtures were separated into CD4$^+$ and CD8$^+$ subsets, however, only the former were active, and the infection was blocked by AZT (Fig. 5, A and B).

Either the Cutaneous T Cell or the Dendritic Cell Can Initiate Productive Infection. The ability of the individual cell subsets to initiate active infection was examined by exposing sorted dendritic cells or T cells to HIV and then recombining them with non–HIV-pulsed T cells and dendritic cells, respectively. Bulk cells and the sorted cell fractions were monitored in parallel during a 7–9 d period by measuring the RTase activity in the culture supernatants. Productive infection was initiated when either the dendritic cells or the T cells were pulsed with virus (Fig. 5 C; DC-HIV and T-HIV), but again both cell types were required for productive infection.

The infection transmitted was also cytopathic. When dendritic cells were pulsed with virus and mixed with T cells that were not pulsed with virus, syncytia formation took place within 3–4 d (Fig. 5 D). As shown in earlier work (12), most of the new viral protein synthesis, as detected by the anti-p24 mAb to an HIV-1 *gag* component, took place in syncytia rather than free cells (Fig. 5 D).

We considered the possibility that small numbers of dendritic cell–T cell conjugates could contaminate the purified cell types and be responsible for virus carriage. However, only rare conjugates were detected under the hemocytometer (0–1.5%) and by FACS® analysis (10, 12). We also depleted conjugated T cells from the dendritic cell preparation by adsorption on magnetic Dynabeads coated with anti-CD2. Depletion with anti-CD2 greatly reduced the infection when applied to mixtures of dendritic cells and T cells (from 10^3 down to <10 HIV copies; data not shown), but not when applied to the purified dendritic cells (Fig. 4 C). Furthermore, magnetic bead depletion did not interfere with the ability of the cells to introduce infectious virus (see Fig. 7, A–C). To minimize the possibility of dendritic cell–T cell conjugate contamination, sorted fractions were routinely magnetic bead depleted.

Infectivity of HIV-Pulsed Dendritic Cells Is Long Lived and AZT Sensitive. To distinguish active infection of the dendritic cell from carryover of virus into the coculture, our approach was to test whether the ability of the virus-pulsed cells to introduce infectious virus was sensitive to AZT treatment. Since reverse transcription can take a day or more to complete (23–25), we studied the infectivity of virus-pulsed cells that had been cultured for up to 1.5 d in the presence or absence of AZT before mixing with non–HIV-pulsed T cells.

First, we carried out experiments to evaluate the extent to which the carryover of AZT (after washing of the cells) could block replication. HIV replication in the cutaneous dendritic cell–T cell environment was sensitive to very low levels of AZT. When skin cell emigrants were exposed to B 11/88 HIV for 90 min and then cultured in the presence of titrated doses of AZT, as little as 0.01 and 0.1 μM AZT significantly inhibited infection at 1.5 and 9 d, respectively

A

B

C
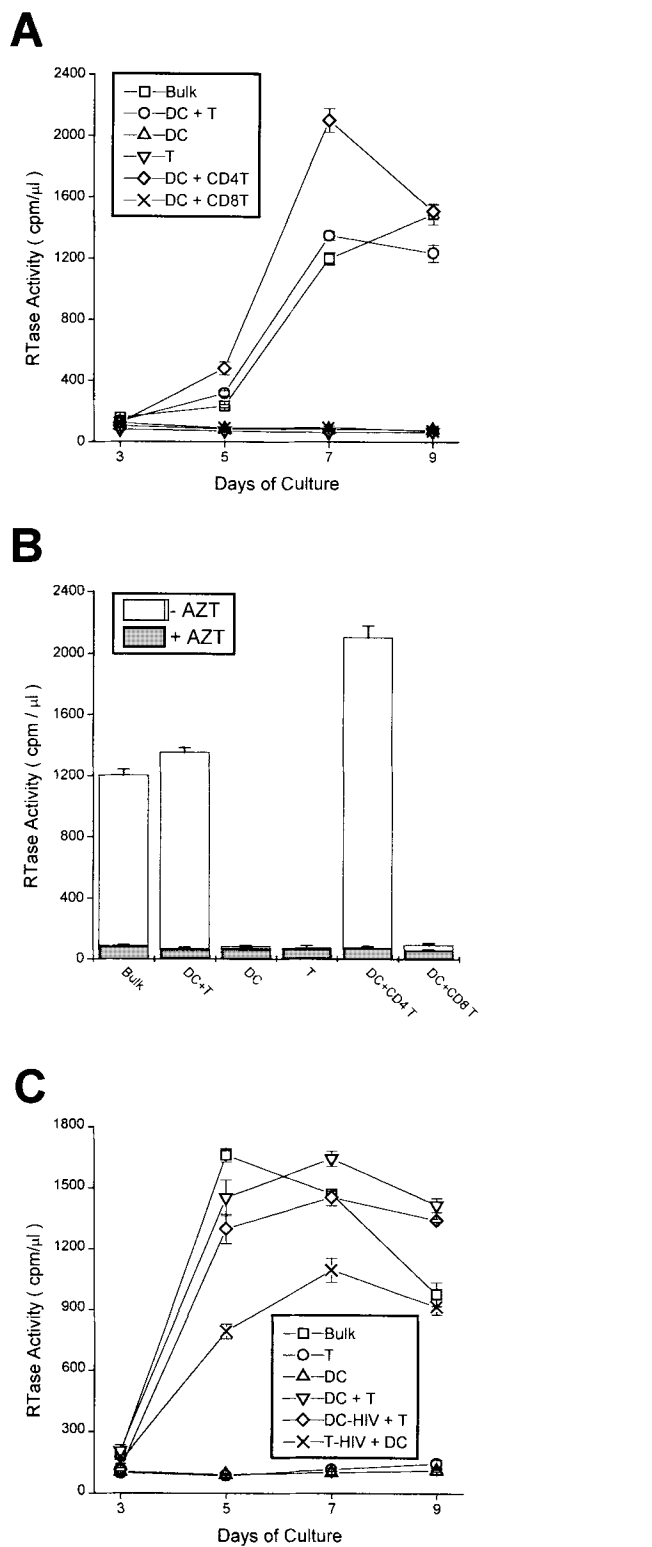
(Fig. 6 *A*). These results underscore the importance of re-moving residual AZT in the coculture experiments. To control for AZT carryover, dendritic cells cultured for up to 1.5 d with or without 10 μM AZT were washed six times in Eppendorf tubes and added to HIV-pulsed skin cell emigrants. Compared to using fewer washes (data not
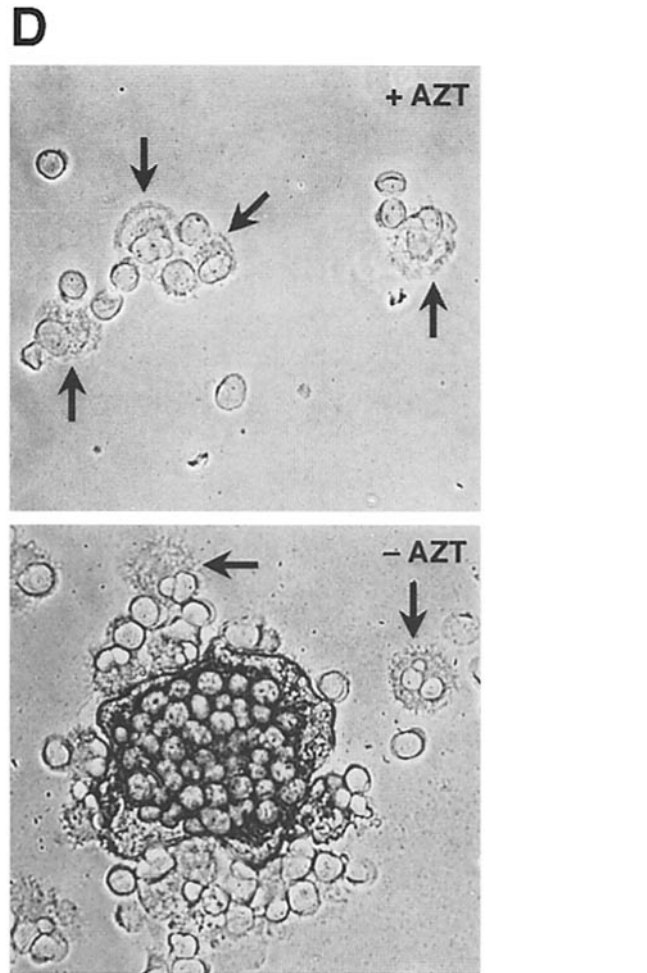
D

Figure 5. Either dendritic cells or T cells can provide infectious virus to the permissive environment created by CD4$^+$ (but not CD8$^+$) T cells and dendritic cells. (*A* and *B*) Free dendritic cells (*DC*) and T cells (*T*) were isolated from the emigrated cell suspension (*Bulk*). A portion of the T cells were sorted into CD4$^+$ (*CD4T*) and CD8$^+$ (*CD8T*) T cells. Un-sorted bulk skin cells, sorted free dendritic cells, sorted T cells, and mix-tures of dendritic cells with either total T cells (*DC* + *T*), CD4$^+$ T cells (*DC* + *CD4T*), or CD8$^-$ T cells (*DC* + *CD8T*) were pulsed with 0.05–0.1 MOI of the NSI B 5/85 isolate with (+) or without (−) AZT treat-ment. The cells were cultured for up to 9 d and the RTase activity was measured. Data are expressed as the mean cpm/μl (± SEM) of culture su-pernatant (*A*). The effect of AZT treatment is shown at day 7 (*B*), but identical effects of AZT were observed at all time points. (*C*) T cells (*T*) and dendritic cells (*DC*) were sorted from bulk emigrated skin cells (*Bulk*). Bulk cells, sorted dendritic cells, sorted T cells, or mixtures of the two (*DC* + *T*) were pulsed with 0.05–0.1 MOI of B 11/88 SI isolate. The cells were washed thoroughly. In the case of the sorted dendritic cells and T cells, HIV-pulsed dendritic cells were also mixed with unpulsed T cells (*DC-HIV* + *T*) and vice versa (*T-HIV* + *DC*). Productive infection was measured by the release of RTase activity into the supernatants over the next 9 d of culture. Representative observations for four similar ex-periments are presented. (*D*) Sorted dendritic cells were exposed to the B 11/88 HIV-1 isolate, mixed with non-pulsed T cells, and cultured with (+) or without (−) AZT for 4 d. Cytospins were immunostained for ex-pression of HIV p24 antigen. Expression of p24 was observed on multi-nucleated syncytia, but not on free dendritic cells (*arrows*). This syncytium is one of the largest observed, containing >35 nuclei. Fields representa-tive of three experiments are shown. ×300.
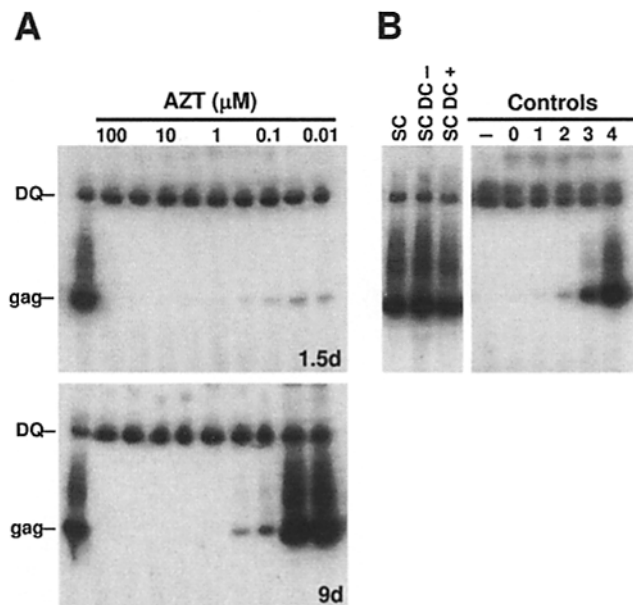
Figure 6. HIV-1 infection of cutaneous dendritic cell–T cell cocultures is sensitive to low doses of AZT. (*A*) Skin cell emigrants were exposed to the B 11/88 isolate for 90 min, washed, and cultured for up to 9 d with or without AZT added to the cultures. The final concentrations of AZT used were 100, 30, 10, 3, 1, 0.3, 0.1, 0.03, and 0.01 μM (every other lane is labeled following this order). DNA was prepared after 1.5 and 9 d of culture and infection monitored by amplification of HIV-*gag* DNA. After 9 d, 0.1 μM AZT significantly blocked infection. Data representative of two identical experiments are shown. (*B*) Dendritic cells were separated by cell sorting and further purified using anti-CD2–coated magnetic beads. The dendritic cells were cultured for 18 h with (DC+) or without (DC−) 10 μM AZT and washed six times in Eppendorf tubes. 3 × 10⁴ cells were added to 9 × 10⁴ HIV B 11/88-pulsed skin cell emigrants. The skin cell–dendritic cell mixtures (SC DC + and SC DC −) and control HIV-pulsed emigrants alone (SC) were cultured for 9 d, DNA prepared, and HIV-*gag* DNA amplified. Similar observations have been made in two separate experiments where the DC were cultured with AZT for 1.5 d before adding to the HIV-pulsed emigrants.

shown), this washing procedure efficiently removed any residual AZT (Fig. 6 *B*).

Consequently, dendritic cells were placed into Eppendorf tubes and exposed to HIV-1 B 11/88 for 90 min in the presence or absence of 10 μM AZT. The cells were washed and cultured (or not) for 18–39 h more with or without 10 μM AZT. The dendritic cells were washed again before being mixed with non–HIV-pulsed T cells, and then productive infection was monitored over the next 7–9 d. The ability of either the dendritic cells or the T cells (not shown) to introduce infectious virus was unaltered if the AZT was removed after 90 min and the cells were mixed immediately after the virus pulse (Fig. 7 *A*). When HIV-pulsed dendritic cells were cultured for 18 h, however, they did not lose infectivity, and it was only partially reduced when AZT was present during the 18-h culture period (Fig. 7 *B*). Infectivity of HIV-pulsed dendritic cells was maintained for 39 h, but the presence of AZT during this culture period ablated the infectivity of the dendritic cells (Fig. 7 *C*). Therefore, even though dendritic cells ap-

parently carry only 10–100 copies of proviral DNA per 5 × 10⁴ cells (see Fig. 4), this low level infection, which is sensitive to AZT, is responsible for the infectivity of these cells (Fig. 7 *C*).

Discussion

Distinctive Features of Cutaneous Leukocytes. Much of the experimental work on HIV-1 infection has been directed to the accessible cells from blood. Blood cells differ in several ways from the cutaneous cells studied here. Conjugates of dendritic cells and T cells are difficult to detect unless antigens or superantigens are added (26–29). In contrast, the cells that emigrate from organ cultures of human skin, including cadaver skin obtained within 36 h of death, form numerous tight conjugates, and these prove to be a critical site for HIV-1 infection (see Figs. 1 and 4, and reference 12). The conjugation of cutaneous dendritic cells and T cells appears to be antigen-independent in the sense that T cell proliferation is minimal (see Table 1, Fig. 2, and references 10, 12). One would anticipate that if antigens were being presented in the conjugate, the T cells would proliferate actively, as occurs in dendritic cell–T cell clusters in the MLR (30) and responses to mycobacteria (26, 31) and superantigens (28, 29). When skin cells are cultured for 3 d, the frequency of T cells in cycle is <2%. At least 10% of the cells are tightly conjugated to dendritic cells, however, as determined by FACS® analyses, by direct observation under the hemocytometer, and in cytospin preparations. These conjugated T cells cannot be stained for the Ki-67 antigen expressed by cells in cycle, and the T cells do not express detectable levels of such activation antigens as HLA-DR, CD25, CD54, CD80, and CD86. Instead, the T cells express a memory phenotype and are competent to proliferate upon stimulation (see Table 1, Fig. 2, and reference 10).

The Infected Dendritic Cell Initiates Viral Replication with CD4⁺ T Cells. As in earlier studies with the III_B isolate, a productive and cytopathic infection took place only with mixtures of dendritic cells and T cells (see Fig. 4–7 and reference 12). Here we show that either cell could be pulsed with HIV-1 and transmit the infection. Although only small amounts of proviral DNA were detected in the dendritic cells (see Fig. 4 *C*), this DNA was not reverse transcribed when AZT was added. This low level of infection of dendritic cells is significant because AZT ablated their infectivity for added T cells. The AZT had to be added to the HIV-pulsed dendritic cells for a sufficient time, about 1.5 d, to fully block reverse transcription (see Fig. 7). We cannot formally exclude a role for small numbers of dendritic cell–T cell conjugates in the dendritic cell population. We were able to deplete T cells efficiently with anti-CD2, however, and this did not reduce the infectivity of the dendritic cell fraction.

We also carried out dozens of experiments in which the infectivity of HIV-1–pulsed memory T cells was compared with HIV-1–pulsed dendritic cells. The results with the T cells were variable, however: in many cases, the virus-
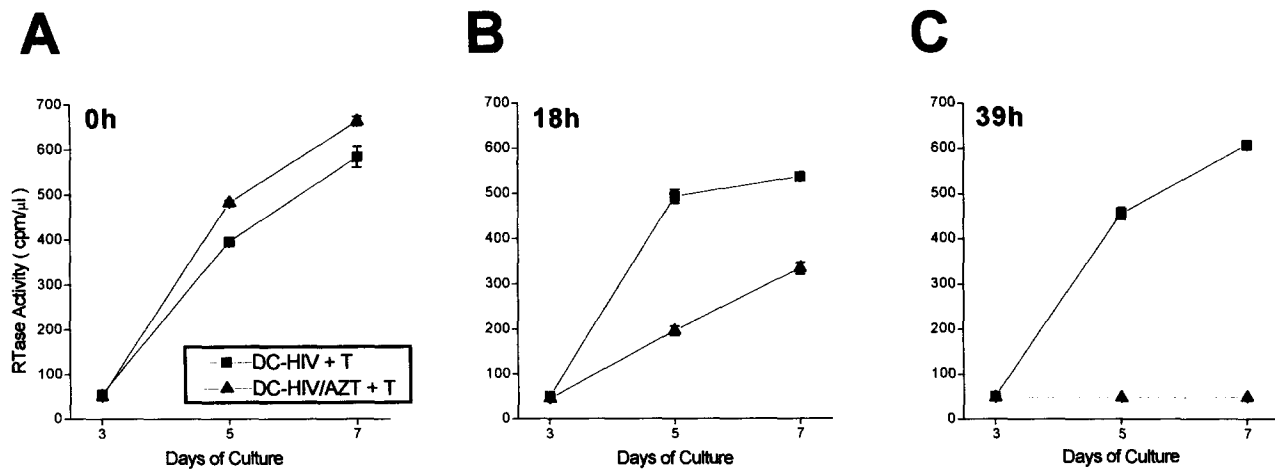
Figure 7. Introduction of infectious virus by HIV-pulsed dendritic cells is blocked by AZT. (*A–C*) T cells and dendritic cells were purified from emigrants by cell sorting followed by magnetic bead depletion. Each population was separately exposed to HIV-1 B 11/88 for 90 min in Eppendorf tubes in the presence (e.g., DC-HIV/AZT) or absence (DC-HIV) of 10 μM AZT. After washing, HIV-pulsed dendritic cells were mixed immediately (*A*, 0 h) with non-pulsed T cells (DC-HIV + T, DC-HIV/AZT + T). HIV-pulsed dendritic cells were cultured for 18 h (*B*) or 39 h (*C*) (without or with AZT), washed, and mixed with non–HIV-pulsed T cells. Control cocultures of HIV-pulsed dendritic cells and T cells with 10 μM AZT in culture (not shown) were included to demonstrate the blocking effects of AZT. Productive infection was monitored for 7–9 d by measuring RTase activity in the culture supernatants. Representative observations of three similar experiments are shown.

pulsed T cell did not maintain infectivity when cultured for 1.5 d, in contrast to dendritic cells (see Fig. 7). We encountered two problems in assessing the behavior of the T cell. First, in contrast to the dendritic cell fraction, the T cell fraction could contain small numbers of conjugates; second, when we tried to remove these with beads (carrying anti-DR antibodies), we often failed to remove the conjugates.

Our findings with skin contrast with earlier studies (32, 33) with blood dendritic cells that had low levels of CD4 (32). In those studies, virus-pulsed dendritic cells also transmitted a vigorous infection to CD4+ T cells or augmented the transmission of infection from infected to uninfected T cells, but (*a*) antigen or superantigen had to be added to the culture, i.e., T cell activation was required; (*b*) the dendritic cells lost most or all of their infectivity if cultured for >12 h before adding T cells; (*c*) the dendritic cells were not incorporated into syncytia; and (*d*) the infectivity of virus-pulsed dendritic cells was not blocked by AZT.

With all isolates tested, HIV-1 p24 antigen is found primarily in syncytia rather than in single cells (see Fig. 5 *D* and reference 12). These syncytia developed only in the presence of both cell types and contain both dendritic cells and T cells. Therefore, conjugates of the two cell types may facilitate syncytium formation, and the latter facilitates virus replication. We have recently found that syncytium formation allows active NF-κB from the dendritic cells to mix with Sp-1 from the T cells, and these two factors within the heterokaryon may accelerate viral replication (34).

Relevance to HIV-1 Infection In Situ. Dendritic cells enter cutaneous lymphatics in substantial numbers, up to 10^5 per h in different species, as reviewed elsewhere (35). Afferent lymph also contains T cells of the memory phenotype (36, 37). Dendritic cell–T cell conjugates have been reported in the lymph from several species (38–41). The

flux of dendritic cells in lymph in the steady state may derive from the dermis, not the epidermis, where cell turnover is very slow (42, 43). However, emigration from the epidermis can occur during stimulation, e.g., during transplantation and explanation (10, 11, 44). During this migration, the dendritic cells markedly upregulate HLA–class II and several adhesion molecules (44, 45). These high levels may mediate binding and partial activation of T cells even in the absence of antigen. During sexual transmission, there may be comparable stimuli for dendritic cell migration and T cell conjugation, and there are allogeneic reactions between donor and host as well.

Although the ability of dendritic cells to transfer virus to T cells is known, a major finding in this paper is that infected dendritic cells can be the starting point, even though their level of infection is low. The ability of infected dendritic cells to carry HIV is relatively long lived, lasting for at least 1.5 d (see Figure 7). <100 copies of proviral DNA per 5 × 10^4 dendritic cells were detected in a dozen experiments of the type shown in Fig. 4. Release of RTase or p24, formation of syncytia, and cell death were all difficult to detect in purified dendritic cells or T cells. Yet when they were mixed, a vigorous cytopathic infection ensued. There are conflicting reports on the extent of active infection of cutaneous dendritic cells in situ (46–48), but some viral nucleic acid is detected in skin (49–53) and in unidentified cells in the mucosa of HIV-infected people (6, 7) and SIV-infected macaques (8, 9).

While the level of infection of dendritic cells and memory T cells in HIV-infected patients is likely to be of the low magnitude described here, both cell types are present in the lymph and can interact there. We therefore hypothesize that dendritic cells and memory CD4+ T cells together represent a site for chronic HIV-1 replication and

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death of memory CD4$^+$ T cells in situ. Conjugates of dendritic cells and memory T cells may represent the milieu for viral replication and T cell death that was recently documented in infected indivdiuals (54, 55). Earlier studies have emphasized virus production in blood and lymph nodes, but it may be important to look in extralymphoid tissues, including mucous membranes, and cells that enter afferent lymphatics.

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