In Vitro Characterization of Purified Human Thymic Dendritic Cells Infected with Human Immunodeficiency Virus Type 1

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In the thymus, dendritic cells (DC) are functionally associated with thymocytes and are recognized to play a major role in the intrathymic differentiation of T cells. Several studies have previously investigated the role of DC during HIV-infection, but the status of thymic DC in HIV-1 pathogenesis remains unclear. In this study, we investigated the susceptibility of purified human thymic DC to HIV-1 infection in vitro. HIV-1 was not detected in cell-free supernatants collected from HIV-infected DC. However, these cultures were shown to transmit HIV-1 infection since coculture with permissive MT4 cells resulted in virus production. The exposure of DC in culture to HIV-1 was shown to promote severe DC morphological changes and killing. We also found that one or several heat labile soluble cytotoxic agents present in the HIV-1-infected DC supernatant mediated the killing of thymocytes. Our observations raise the possibility that (1) the HIV-1-induced DC killing, (2) the capacity of DC to transmit viral infection, and/or (3) the release of HIV-1-mediated cytotoxic agent(s) from DC may contribute to AIDS pathogenesis in vivo.

INTRODUCTION

Clinical and immunological manifestations of AIDS are known to be associated with HIV infection. The most consistent and striking observation of HIV infection is a progressive depletion of CD4+ T cells (Klatzmann et al., 1984). The dysfunction and quantitative depletion of CD4+ T cells have been proposed to occur by a wide range of distinct direct and/or indirect mechanisms (as reviewed in Levy, 1993). Recently, a simple steady-state model has been proposed in which infection, cell death, and cell replacement are in balance during the period prior to the onset of AIDS (Coffin, 1995; Ho et al., 1995; Wei et al., 1995). This suggests that an imbalance of such a dynamic process, either by an increase of CD4+ T cell mortality or a decrease in CD4+ T cell replenishment, could result in a decrease of the CD4+ T cell population. Recent findings support rather the view that AIDS is primarily a consequence of continuous rapid high-level replication of HIV-1 leading to virus- and immune-mediated killing of CD4+ lymphocytes. However, indirect mechanisms leading to a decrease in the level of CD4+ mature T cell replenishment might influence the period of HIV-related disease prior to the onset of AIDS.

The human thymus is an active organ that plays a key role in the maturation, differentiation, and function of the T cell immune system (Ritter and Boyd, 1993). The presence of HIV infection and severe thymic abnormalities with involution and disruption of normal architecture have been noted at necropsy in patients dying with AIDS (Joshi et al., 1986; 1990; Nezelof, 1992; Mano and Chermann, 1991). In addition, HIV infection of severe combined immunodeficiency (SCID) mice implanted with human thymic tissue resulted in marked depletion of thymocytes, productive infection, and destruction of stromal elements (Aldrovandi et al., 1993; Bonyhadi et al., 1993; Stanley et al., 1993). These observations suggested a possible role of the thymus in the pathophysiology of AIDS. Thymocytes at different stages of cell maturation and nonlymphoid cells have been previously shown to be susceptible to HIV infection both in vivo and in vitro using SCID mice reconstituted with human thymus (Stanley et al., 1993; Tremblay et al., 1990; De Rossi et al., 1990; Schnittman et al., 1990; Valentín et al., 1994; Sandborg et al., 1994). One of the nonlymphoid cells targeted by HIV has been proposed to be thymic dendritic cells (DC) (Valentín et al., 1994; Müller et al., 1993). DC represent a family of cells which are found in small numbers in nonlymphoid and lymphoid tissues (Steinman, 1991). DC express a high level of MHC class II molecules and are recognized to be potent APC for a variety of T cell-dependent immune responses (Steinman, 1991; Bjorck et al., 1985). Human thymic DC are narrow-derived leukocytes which are intimately associated with thymocytes (Landry et al., 1989). The involvement of DC in thymocyte maturation is generally accepted, although their precise influence is still being debated.

DC from several tissues have been analyzed for in vivo and in vitro susceptibility to HIV-1 infection (review in...
Cameron et al., 1996). To date, it is not yet clear whether DC can support fully productive replication of HIV-1 because of conflicting data. Apparent contradictory results seem to depend upon the composition of the cell population studied which differ according to the method of purification or to whether in vivo populations were analyzed. Productive HIV-1 infection was demonstrated in skin DC in in vivo studies (Langerhans cells (LC)) (as reviewed in Dezutter-Dambuyant and Schmitt, 1994). Similarly, results from in vitro experiments demonstrate that productive HIV-1 infection was also observed in conjugates of DC and T cells derived from normal skin but not in purified LC largely free of T cells (Pope et al., 1994). Results from in vitro studies have demonstrated that blood DC can be readily infected by HIV-1 (Patterson et al., 1991; Langhoff et al., 1991), whereas a recent study suggests that blood DC, like LC, do not sustain productive infection, but rather transmit cytopathic infection to stimulated T cells (Cameron et al., 1992).

To assess the role of thymic DC in HIV-1 pathogenesis, we studied the susceptibility of purified human thymic DC to HIV-1 infection in vitro. We report here that HIV-1 infection of highly purified thymic DC does not lead to the release of detectable viral particles in culture supernatant. However, the infected DC cultures were found to be associated with fully infectious viral particles even in long-term cultures and to exhibit profound cell morphological changes and mortality. In addition, we show that HIV-1-infected DC failed to promote thymocyte stimulation. Our results demonstrate that this phenomenon is mediated by one or several soluble thymocyte killing factor(s) released in the supernatant from HIV-1-infected DC.

MATERIALS AND METHODS

Cell lines and preparation of purified thymic dendritic cells

UHC-1, a promonocytic cell line chronically infected with the IIIB strain of HIV-1 (Boulerice et al., 1990) and MT4, a human T-lymphotropic virus type-1 (HTLV-I)-transformed T-lymphoid cell line (Harada et al., 1985), were kept in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated FCS. Cos-7, an African green monkey kidney cell line transformed by an origin-defective mutant of simian virus 40 (Gluzman, 1981) was maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FCS. Cells were incubated at 37° in a 5% CO₂ humidified atmosphere.

Dendritic cells were isolated according to the Double Step purification method described previously (Beaulieu et al., 1995). Briefly, unfractionated human thymic cells were first depleted of CD2⁺ reacting thymocytes by incubation with neuraminidase-treated sheep red blood cells followed by a Ficoll separation. The remaining cells were separated according to their density on a 50% Percoll gradient. Cells were then further enriched by negative depletion using a cocktail of unconjugated monoclonal antibodies containing anti-CD2, -CD3, -CD5, -CD7, -CD8, and -CD14. Reactive cells were fixed to magnetic beads and removed using a magnetic separator. Purified dendritic cell were maintained at 2 × 10⁶ cells/ml in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin, 25 mM HEPES, 4 mM glutamine, 10 mM 2-mercaptoethanol, and 2 mM sodium pyruvate and incubated at 37° in a 5% CO₂ atmosphere. This purification method allows the reproducible recovery of cell populations as characterized in Table 1. Unfractionated thymic cell populations were maintained at high concentration in the same medium at 4° before the assays.

Virus stock and HIV-1 infection

Stocks of the Tropic HIV-1 IIIB strain were produced from the UHC-1 cell line (gift of Dr. M. Wainberg, Lady Davis Institute for Medical Research, Montréal, Québec). A stock of the macrophage tropic HIV-1 BaL strain was produced by transfecting Cos-7 cells with pIIIB-BaL (gift of Dr. Bryan Cullen, Durham, NC; Hwang et al., 1991). Each viral stock was obtained from concentrated cell-free culture supernatants filtered through a 0.22-µm syringe-loaded filter unit (Costar, Cambridge, MA) and titrated by end-point dilution using the MT4 cell line (IIIB) or by measurement of reverse transcriptase (RT) activity (BaL). HIV-1 (IIIB) viral stocks were heated at 58° for 50 min for assays using heat-inactivated virus (Harada et al., 1985). Lack of infectivity of the heat-inactivated virus was tested on HIV-sensitive CD4⁺ MT4 cells.

As indicated, freshly purified DC and unfractionated thymic cells were mock-infected or infected with viral stocks at m.o.i. of 0.1 TCID₅₀/cell (IIIB) or on the basis of identical RT activity (BaL). After an adsorption period of 4 to 6 hr at 37°, in a 5% CO₂ atmosphere, cells were extensively washed with PBS to remove nonadherent viruses. Infected or mock-infected DC and unfractionated thymic cells were then resuspended at 2 × 10⁶ cells/ml in supplemented RPMI 1640 containing 10% filtered supernatant collected from a 48-hr PHA-stimulated PBL culture. Cell suspensions were subsequently distributed in 24-well cell culture plates (1 ml/well) (Falcon, Lincoln Park, New Jersey) or in 15-ml tubes for the following assays.

Detection of viral particles

To detect viral particle production, cell supernatants were tested for the presence of reverse transcriptase (RT) activity and p24 (capsid antigen) as follows. Two-hundred fifty microliters of supernatants from triplicate cultures of DC or unfractionated thymic cells were collected each day and replaced with fresh medium. Virus-specific RT activity in each supernatant was measured as described (Lee et al., 1987). Release of capsid anti-
gens was determined by detection of p24 in the culture supernatant, using an enzyme-linked immunosorbent assay kit (ELISA HIVAG-1, Abbott Laboratories, Abbott Park, IL). The sensitivity of the assay is 1–2 pg/ml.

In reinfection assays, 250 µl of supernatant was collected, centrifuged at 3000 rpm, and filtered. At Days 2, 4, 6, 7, and 11 the filtered supernatants were added to 2 × 10⁶ MT4 cells/ml cultured in 24-well plates. In coculture assays, 2 × 10⁶ MT4 were directly added to noninfected or to infected-DC at Days 2, 6, and 11 following in vitro culture on 24-well plates. Viral replication was examined for up to 28 days according to the following procedures; 250 µl of supernatant from triplicate cultures were collected each day and replaced with fresh medium. MT4 infection was monitored by measurement of RT activity and p24 in each supernatant as well as for the presence of syncytium formation.

Cell survival kinetics

Cell viability was monitored daily in uninfected DC, unfractonated thymic cells, or in DC infected with either live or heat-inactivated HIV-1 virus by trypsin blue exclusion assay. Cell viability was also monitored in 24-hr uninfected DC or in unfractonated thymic cells treated with filtered supernatants collected from autologous uninfected or infected DC at Day 1 or from allogenic uninfected or infected DC at Day 6.

FACS analysis of infected and uninfected dendritic cells

Phenotypic analysis of infected and uninfected DC was determined by direct or indirect immunofluorescence with anti-CD1, -CD2, -CD3, -CD4, -CD5, -CD7, -CD8, -CD11b, -CD14, -CD20, -HLA-DR monoclonal antibodies (mAbs) (Becton Dickinson, Mountain View, CA; Coulter Immunology, Hialeah, FL; and Ortho Diagnostic, Raritan, NJ) as described (Beaulieu et al., 1995). Briefly, 2 × 10⁵ cells were incubated on ice for 1 hr with a saturating amount of the appropriate mouse anti-human mAb. The cells were subsequently incubated on ice during 30 min with a saturating amount of FITC-conjugated goat anti-mouse IgG (Gibco/BRL). MAb-reactive cell distribution was analyzed using a FACStar at Days 0 and 2 following purification (Becton Dickinson, Mountain View, CA) with the Consort 30 program.

In situ hybridization analysis

The cell pretreatment and in situ hybridization (ISH) were performed as described (Haase et al., 1984; Cox et al., 1984) with minor modifications. Briefly, infected and uninfected DC samples were centrifuged and the cell pellets were rinsed and resuspended in PBS buffer. Duplicate samples made of 2 × 10⁵ cells were cytocentrifuged at 400 rpm for 5 min in a Cytospin. The cells were fixed in a mixture of methanol/acetic acid (3:1) for 15 min at 4°, dried, and pretreated with proteinase K and acetic anhydride as described. They were then hybridized overnight at 55° with 35S-labeled sense or antisense RNA probes transcribed from the plasmid pGEM3-vpu which harbors the HIV-1-specific vpu gene under the control of the Sp6 and T7 phage RNA polymerase promoter (Terwilliger et al., 1989). The anti-sense probe used allows detection of unspliced and singly spliced viral RNAs. The hybridization mixture (20 µl/slide) consisted of 50% formamide, 0.6 M NaCl, 1× Denhart solution, 10 mM Tris–HCl (pH 8), 1 mM EDTA, 10% Dextran sulfate, 0.5 mg/ml tRNA, 100 µg/ml poly(A) RNA, 1 mg/ml BSA, 10 mM dithiotreitol, 10 mg/ml salmon sperm DNA, and the equivalent of 5 × 10⁵ cpm of the labeled probes. After hybridization, the slides were rinsed several times in a buffer containing 10 mM Tris (pH 8), 1 mM EDTA, 0.6 M NaCl, and 50% formamide at 55° followed by several washes at room temperature in the same buffer without formamide. Finally, the slides were rinsed in 1 SSC (0.15 M NaCl, 0.015 M sodium citrate), dehydrated, and covered with NTB2 Kodak nuclear emulsion. After a 10- to 14-day exposure, the slides were developed in D19 developer (Kodak) and fixed in 30% sodium thiosulfate. The specific signal was evaluated by quantifying the number of silver grains associated to the cells and subtracting the background labeling.

Immunocytochemistry reactions to detect the presence of HLA-DR antigens were performed on similar cytospin cell preparations that were fixed in cold acetone for 30 min. Slides were treated with 0.3% hydrogen peroxide in PBS to block endogenous peroxidase activity for 30 min. Cells were subsequently washed twice in PBS and incubated at room temperature with a saturating amount of HLA-DR mAb for 30 min. Samples were washed three times in PBS, incubated with biotinilated goat anti-mouse IgG (H + L) (Caltag Laboratories, San Francisco, CA) at 1:500 for 30 min, washed in PBS, and incubated with peroxidase-conjugated streptavidin 1:1000 for 30 min. The reaction was revealed with 0.05% diaminobenzidine (DAB) and 0.01% hydrogen peroxide in a 0.05 M Tris buffer, pH 7.6. In control samples, cells were treated in a similar fashion but in the absence of primary antibody. Cells were counter stained with hematoxylin, mounted in Permount, and examined with a Zeiss microscope.

Electron microscopy analysis

Transmission electron microscopy (EM) was performed on 10 × 10⁵ cells as described in Beaulieu et al. (1995). Briefly, infected or uninfected cells were fixed with 2.5% glutaraldehyde in Sorensen buffer (0.1 M sodium phosphate, pH 7.2) for 1 hr at 4°. After washing with Sorensen buffer, cells were postfixed in 2% osmium tetroxide before dehydration in graded ethanol and embedded in Epon (JBEM Services, Pointe-Claire, Dorval, Canada). Ultrathin sections were stained with lead Reynold and uranyl acetate (Fisher). Cells were examined with a Philips EM-300 electron microscope.
Statistical analyses for ISH and EM were performed on data obtained from, respectively, two and three independent experiments. We have used the z-test for comparing two proportions and have calculated the associated $P$ value to evaluate the statistical significance (Moore and McCabe, 1993).

### Accessory function assay

The ability of uninfected or infected DC to induce thymocyte proliferation was performed as described in Beaulieu et al. (1995). Briefly, total autologous thymic cells were incubated with or without graded doses of concanavalin A (Con A) (St. Louis, MO) ranging from 0.5 to 10 $\mu$g/ml during 72 hr at 37°C in the presence or absence of 24-hr-cultured purified thymic DC at a 10:1 cell ratio ($3 \times 10^5$ autologous thymic cells for $3 \times 10^4$ purified DC per well) in a 96-well round-bottomed microtiter plate (Linbro, Flow Laboratories). DNA synthesis was monitored by directly adding 0.5 $\mu$Ci $[^3H]$thymidine-methyl (6.7 Ci/mmol, Du Pont Canada Inc., NEN Products, Montréal, Canada) to the culture for 18 hr. Cells were harvested on glass fiber filters (Skatron Titterteck Cell Harvester) and the level of cellular $[^3H]$thymidine-methyl incorporation was evaluated in the presence of scintillation liquid Econofluor-2 (Du Pont Canada Inc.) in a scintillation counter (Beckman LS-600 SC). Each sample was performed in triplicate. As indicated, 24-hr cultured DC were also incubated 24 hr prior to the accessory function assays with filtered cell culture supernatants collected at Day 1 after infection and/or purification from autologous DC.

### RESULTS

Evaluation of viral production in HIV-infected DC population

Unfractionated thymic cells or populations of freshly purified human thymic DC containing more than 80% of CD2−/HLA-DR+ cells were infected either with laboratory HIV-1 isolates (IIIB or BaL strains) or with heat-inactivated HIV-1 (IIIB) in order to evaluate the susceptibility of cells to HIV-1 infection. The cytofluorometric analysis of DC at Days 0 and 2 following infection and/or purification are described in Table 1. No significant difference was observed in the distribution of mAb reactive cells between uninfected and infected DC even after 2 days of culture. During the culture period, ranging from Days 0 to 11, supernatants were collected daily from uninfected or infected DC populations and analyzed for the presence of RT activity and p24 Gag proteins. No viral production was detected in infected cultures either by measurement of RT activity or by using a very sensitive p24 ELISA assay (results not shown). In contrast, as measured by p24 assay, HIV-1 IIIB-infected unfractionated thymic cells exhibited viral production that reached a maximum at Day 4 postinfection (results not shown).

In order to determine if infectious viral particles were present in cell supernatants, we exposed the highly HIV-1-sensitive cell line, MT4, to cell-free supernatants collected at Days 2, 4, 6, 7, and 11 from uninfected, HIV-1 (IIIB strain), or heat-inactivated HIV-1-infected DC. MT4 cells were monitored daily for cytopathic effects and RT activity, as well as for the presence of p24 antigen in cell supernatants. Viral replication was not detected in MT4 cells infected with any culture supernatant up to 28 days (data not shown). These results indicate that infectious viral particles were not detectable in the supernatant fluids of HIV-1-infected DC populations.

To determine whether HIV-infected DC could transmit infection, we added HIV susceptible MT4 cells to DC populations at 2, 6, or 12 days postinfection with live or heat-inactivated HIV-1 (IIIB). RT activity in the supernatants was measured everyday or every 2 days. Viral replication was detectable since MT4 cells, in coculture with HIV-1-infected DC, exhibited typical syncytiuim formation (data not shown), and RT activity was present in the supernatant (Fig. 1). In contrast, viral replication was not observed in MT4 cells cocultured with heat-inactivated HIV-1-infected DC (data not shown). Maximum RT activity values were observed at Day 4 when MT4 cells were cocultured with 6 or 12 days postinfected DC populations. Peak RT activity was detected later (Day 8) when MT4 cells were cocultured with a 2 day postinfected DC population. These data indicate that HIV-infected DC do...
FIG. 1. Detection of RT activity in supernatants of HIV-1-infected DC populations cocultured with permissive MT4 cells. MT4 cells were added to uninfected (*) or to HIV-1-infected DC populations at Days 2 (− △ −), 6 (− ■ −) or 12 (− ● −) postinfection. Supernatants were monitored for RT activity, as indicated, every 1 or 2 days. Results represent mean values from two independent experiments with SE values of less than 10%. A statistically significant difference in the time of apparition of RT peak value (Days 4 or 8) was observed between MT4 cocultured with a 2 day- and MT4 cocultured with a 6- or 12-days postinfected DC.

not release detectable levels of infectious viral particles in the supernatant. However, infectious viral particles are present in association with DC over a period of at least 12 days since infection can be transmitted following a cell-to-cell contact upon cocultivation of DC with a CD4+ T cell line. Moreover, the extent of viral transmission by DC seems to correlate with the time of culture postinfection, suggesting that viral replication may be an important factor.

Detection of viral RNA by in situ hybridization

HIV-1-infected DC cultures were analyzed by ISH to determine the percentage of cells harboring specific viral RNAs. No cell labeling was observed in HIV-1 (IIIb)-infected DC collected immediately after the adsorption period (Table 2), indicating that genomic RNAs from virions still present at the cell surface were not detected in our ISH conditions. However, as shown in Fig. 2c and Table 2, a total of 18% of cells in the DC population collected 16 hr postinfection were labeled with the antisense probe. In contrast, no signal was detected in HIV-1-infected DC populations hybridized with the sense probe (Fig. 2a). Similarly, no silver grains were observed in uninfected DC populations hybridized with either the antisense (Fig. 2b) or the sense probe (data not shown). Interestingly, a statistically significant increase in the proportion of positively labeled HLA-DR+ cells was observed between 16 and 48 hr postinfection (18 vs 30.5%) (P = 0.0035) (Table 2).

As evaluated by the examination of ISH slides and a parallel immunoperoxidase staining of samples from the same DC preparation (Table 2), cell populations are composed of approximately 49 and 8% of single cells showing, respectively, typical dendritic (HLA-DR+) and thymocytic (HLA-DR−) morphology. The remaining cells (43%) represent complexes of DC (31%) and thymocytes (12%) (DC + T). Surprisingly, cells which were found positive by ISH, were not randomly distributed among these subsets. At 16 hr postinfection, only cells exhibiting DC morphology were found positive by ISH. The proportion of positive cells among nonthymocyte-associated DC at 16 hr postinfection (10.5%) is not statistically different from the proportion observed at 48 hr postinfection (9.0%) (P = 0.617). However, the proportion of ISH-positive DC present in DC + T complexes at 48 hr postinfection (17.0%) is significantly higher than the proportion observed at 16 hr postinfection (7.5%) (P = 0.0038). Moreover, at 48 hr postinfection, nonthymocyte-associated DC which composed the majority of cells in the DC population (49/80: 61%) represented only 34.6% (9/26) of the ISH-positive DC. In addition, cells with thymocyte morphology were only found positive in DC + T complexes and were only detected at 48 hr postinfection. The mean number of silver grains (from 8 to 15 grains) in thymocytes was smaller than in DC within these complexes (>15 grains). The non-DC-conjugated thymocytes, which represent 8% of total cells in our DC preparation, were all found negative.
In conclusion, the enhancement of ISH signal observed from 16 to 48 hr postinfection suggests that viral gene expression occurs in DC culture. Moreover, this analysis indicates that, after 48 hr postinfection, HIV positive cells are significantly associated with complexes of DC and thymocytes.

HIV induces cell morphology changes and mortality in dendritic cells

To determine the cytopathic potential of HIV-1 on DC, we performed cell viability and ultrastructural morphological analyses on infected DC. Figure 3 shows that 65% of the initial cell number was present in both uninfected and heat-inactivated DC populations after 11 days of culture as evaluated by trypan blue exclusion cell counts. In contrast, rapid cell death was observed in DC infected with both IIIB (Fig. 3) and BaL HIV-1 strains (data not shown). By Day 3, only 60% of the initial cell number remained viable in infected DC cultures compared to 85% in uninfected DC or in heat-inactivated virus-treated DC cultures. Between Days 0 and 3, the calculated mortality rate in infected DC was 2.8-fold higher than in the noninfected DC cultures. After 11 days, only 40% of cells remained viable in HIV-1-infected DC populations. These data demonstrate that the presence of HIV-1 induces cytopathic effects in DC cultured in vitro. To determine if the cytopathic effects were due to the presence of a toxic compound in cell supernatants, we added supernatants collected from HIV-1-infected DC to uninfected DC. The addition of supernatants collected at Days 1 or 6 from uninfected (not shown) or from HIV-1-infected DC did not significantly increase the mortality of uninfected DC (Fig. 3). These results indicate that the cytopathicity observed in HIV-1-infected DC cultures does not result from the presence of a soluble cellular- or viral-derived toxic product in supernatants.

Uninfected cells or cells infected with live or heat-inactivated HIV-1 IIIB were examined by electron microscopy. EM analysis also revealed a marked morphological change in cells present in the HIV-1-infected DC culture as compared to cells in uninfected or in cultures infected with heat-inactivated HIV-1. At each time point following the adsorption period, the proportion of cells showing vacuolization in HIV-1-infected DC was significantly higher than in uninfected or in heat-inactivated HIV-1-infected DC (all P values of less than 0.001). At 40 hr postinfection, 20% of cells showed the presence of cytoplasmic vacuolization (Fig. 4 and Table 3). The percentage of cells showing vacuolization as well as the size and number of these vacuoles increased progressively throughout the infection (Figs. 4c to 4f). At Day 6, approximately 50% of cells showed the presence of vacuoles and approximately 70% of these cells contained vacuoles which occupied almost all the cytoplasmic area (Fig. 4f). In uninfected or heat-inactivated HIV-1-infected DC at Day 6 of culture, for example, only approximately 10% of cells exhibited vacuolization and all vacuoles were of small size. Taken together, these data show that even in the absence of detectable viral production, the presence of HIV-1 induces cell death and profound morphological changes that could be causally related to cell death.

Effect of HIV infection on DC accessory function

In vitro studies have demonstrated that human thymic DC exhibit an efficient accessory function on the proliferation of
Con A-stimulated mature thymocytes (Beaulieu et al., 1995; Landry et al., 1990). To further assess the role of thymic DC in HIV pathogenesis, we studied the ability of in vitro HIV-1 IIIB-infected DC to function as accessory cells.

FIG. 2. In situ hybridization of DC populations infected with HIV. Photomicrographs show cells from (a) HIV-1-infected DC populations labeled with control HIV-1 sense probe, (b) uninfected DC populations labeled with antisense HIV-1 probe, and (c) infected DC populations at 2 days postinfection labeled with anti-sense HIV-1 probe. Magnification 200×.

FIG. 3. DC survival kinetics. Viable cells were monitored daily by trypan blue exclusion cell counts. Results represent mean values from three independent experiments with SE of less than 5%. Statistically significant differences were observed in the mean values of the number of cells present in HIV-1 (IIIB)-infected populations (○) compared to those in uninfected (*) or in DC treated with heat-inactivated HIV-1 (△) or with supernatant collected from allogeneic HIV-1-infected DC (□) at Day 1 postinfection. Uninfected DC were also treated with supernatants collected from HIV-1 IIIB- (–) or heat-inactivated HIV-1- (–) infected allogeneic DC at Day 6 postinfection.

As exemplified in Fig. 5A, the [3H]thymidine incorporation values in the thymocyte proliferation assay using DC cultured for 24 hr or infected with heat-inactivated virus ranged from 2,000 to 90,000 cpm with a maximum value in populations treated with 5 μg/ml of Con A. In contrast and as observed in 10 independent series of experiments, no significant variation of the thymidine incorporation from the basal level was observed when 24-hr HIV-1-infected DC were added to thymocytes in similar assays. These results indicate that the presence of infectious HIV-1 completely prevents the effect of DC on the proliferation of thymocytes.

As previously published (Beaulieu et al., 1995), purified thymic DC do not represent an homogenous population. Indeed, a CD4+ cell subset represents approximately 20% of the HLA-DR+ cells. In order to investigate their eventual role in the apparent lack of thymocyte proliferation in the DC accessory function assay, we purified in parallel from the same thymus, DC including CD4+ cell subset, and DC mainly depleted of CD4+ cells. As showed in Fig. 5B, uninfected DC including or not the CD4+ DC subset induced similar levels of thymocyte proliferation. No thymidine incorporation was observed when thymocytes were added to HIV-1-infected DC either containing the CD4+ DC subset or depleted of it. These data indicate that accessory function of both DC populations are similarly affected by the virus, suggesting that CD4 expression on DC cell surface is not a prerequisite for the absence of thymocyte proliferation.

As shown in coculture experiments, infected DC were able to transmit HIV to permissive MT4 cells (Fig. 1). Trans-
mission of HIV-1 from infected DC to thymocytes may interfere with cell proliferation in the DC accessory function assays. In addition, the presence of HIV-1 was shown to promote DC morphological changes and cell killing (Fig. 3).

Thus direct killing of DC could explain partly the inability of DC to stimulate thymocyte proliferation in the assays discussed above. In order to determine if such an effect is mediated by infected cell or by a non-cell-associated fac-
TABLE 3
Vacuolization in Uninfected and in HIV-1-Infected DC Populations

<table>
<thead>
<tr>
<th>DC population treatment</th>
<th>Time following purification†</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Uninfected DC</td>
<td>1</td>
</tr>
<tr>
<td>DC + HIV-1 (IIIB) heat-inactivated</td>
<td>(−/+</td>
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<tr>
<td>DC + HIV-1 (IIIB)</td>
<td>(+)</td>
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*a Mean percentage was calculated from EM observation of at least three independent experiments based on 100 cells.
*b Represents the mean number of vacuoles: −, no vacuole; +, 1 to 10 vacuoles; ++, 11 to 30 vacuoles; ++++, >30 vacuoles per cell.
*c Freshly purified cells were infected as indicated and submitted to EM analysis after the period of adsorption (0) or at the indicated times postinfection.

tor(s), we collected supernatants at 24 hr from uninfected DC or from DC infected with heat-inactivated or infectious HIV-1 IIIB. Filtered cell-free supernatants were used to resuspend pellet of autologous uninfected 24-hr-purified DC which were then incubated for an additional 24 hr prior to the functional assay. As exemplified in Fig. 5C, supernatant collected from uninfected DC or from DC infected with heat-inactivated HIV-1 do not interfere with the ability of uninfected DC to stimulate thymocyte thymidine incorporation (maximum thymidine incorporation values of 78,000 and 84,000 cpm, respectively, in the presence of 2.5 μg/ml of Con A). However, as observed in three independent series of experiments, the addition of supernatant collected from HIV-1-infected DC in the functional assays interfere with the ability of uninfected DC to stimulate thymidine incorporation in thymocyte. As demonstrated above, infectious viral particles were not present in supernatants collected from HIV-1-infected DC. These findings indicate that de novo viral infection of DC or thymocytes in these assays are unlikely to be responsible for the observed absence of thymocyte proliferation. In addition, these results indicate that the reduction of thymocyte proliferation is mediated by one or several soluble factors present in the supernatant of HIV-1-infected DC. The filtered-supernatants collected from HIV-1-infected DC were incubated at 58° for 50 min. As presented in Fig. 5C, an increase in the level of thymidine incorporation was observed when uninfected DC were incubated with heat-treated as compared to non-heat-treated filtered supernatants collected from infected DC. These data indicate that the factor(s) present in the HIV-1-infected DC supernatant and which affect thymocyte proliferation is heat-labile.

Thymocyte killing factor(s) released from HIV-1-infected DC

The soluble factor(s) present in the HIV-1-infected DC supernatant may interfere with DC accessory function by targeting uninfected thymocytes, uninfected DC, or both cell types. For that reason, we monitored using trypan blue exclusion assays, viability of both cell types treated with supernatants collected from uninfected DC, or from DC infected with heat-inactivated or IIIB HIV-1 strain at Days 1 and 6 postinfection and/or following purification (Figs. 3 and 6; similar results obtained but not shown for BaL strain). As previously mentioned, the addition of supernatants collected from any of these cultures to uninfected DC did not have significant effect on cell viability (Fig. 3). Surprisingly, the addition of supernatants collected either at Day 1 (Fig. 6) or Day 6 (data not shown) from HIV-1-infected DC to uninfected thymocytes dramatically decreased their viability. In contrast, nontreated uninfected cells or uninfected thymic cells treated with supernatants collected from uninfected DC (data not shown) or from DC infected with heat-inactivated virus exhibited similar survival kinetics (Fig. 6). These data clearly indicate that the absence of thymocyte proliferation observed upon addition of HIV-1-infected DC supernatant results from the killing of thymocytes.

DISCUSSION

In the present study, we investigated the susceptibility of purified human thymic DC to in vitro HIV-1 infection. We showed that infectious viral particles were not present in supernatants of HIV-1-infected DC as determined by the absence of RT activity, p24 antigen detection, and reinfection in assays using a highly sensitive HIV-1 permissive cell line (MT4). Despite the absence of detectable viral particles in supernatants, thymic DC were found to be associated with fully infectious HIV-1. The presence of infectious particles in infected DC was demonstrated by productive infection of MT4 cells in coculture assays.
Thymic DC described in the present paper are composed of 80 to 90% HLA-DR^+ cells. As previously reported, the remaining 10 to 20% of cells which are HLA-DR^- are also negative for several surface markers tested including CD1a, CD3, CD4, CD8 (Beaulieu et al., 1995). Among HLA-DR^+ cells, different phenotypic subsets were further defined based, for example, on the expression of the CD4 receptor. Valentin et al. (1994) have reported that among triple negative (TN: CD3^- , CD4^- , CD8^-) cell populations purified from the human thymus, a CD1a^- cell subset, presumably thymic DC, has been reported to be permissive to HIV-1 infection. Such infection was reported to be dependent on the presence of very low levels of CD4 receptor at the cell surface. Our results clearly demonstrate that DC populations that probably include similar CD4^- expressing cells do not produce virions into the supernatants. This indicates that virion productive cells in the TN cell population reported by Valentin et al. (1994) may not correspond to DC and represents possibly CD3^-, CD4^low/- , CD8^- immature thymocytes.
Conflicting data have been reported regarding viral production in in vitro HIV-1-infected DC purified from different tissues. However, as DC purification methods were improved to eliminate contaminating cells, a new picture has emerged suggesting that highly purified infected DC may not efficiently produce viral particles in supernatants but still can transmit viral infection. Recent data concerning HIV-1 infection of in vitro-purified blood DC, have shown that these cells do not produce HIV but were reported to be associated with infectious particles (Cameron et al., 1992). Moreover, a recent report has shown that the virus can be efficiently transferred to antigen responding cells although DC need not be infected to transmit the virus (Cameron et al., 1994). Skin DC, purified in vitro and shown to be largely free of T cells, were recently reported to support viral entry as indicated by the presence of HIV-1 gag-containing sequences. However, they did not support productive infection as long as the DC were separated from skin T cells by sorting (Pope et al., 1994).

Viral replication in thymic DC cultures is indirectly suggested by results of coculture experiments and ISH analyses. Viral RNAs detected at 16-hr postinfection in HLA-DR$^+$ cells by ISH analysis may result in part from genomic viral RNAs. However, the number of ISH-positive cells significantly increased between 16 and 48 hr postinfection and moreover at 48 hr postinfection, a larger proportion of HIV-positive cells was found in association with DC-thymocyte complexes (DC + T). The increase in the proportion of HIV-positive cells between 16 and 48 hr postinfection may represent the detection of infected cells having undetectable levels of viral RNA at 16 hr. Alternatively, but more likely, this increase may reflect viral gene expression and a certain extent of viral spread among the DC population. We cannot exclude from our data that the contaminating immature thymocytes, phenotypically CD1a$^-$ TN, might support HIV-1 replication. However, the thymocytes which are positive for viral RNAs are exclusively those which are present in DC + T complexes and are detected only at 48 hr postinfection. Valentin et al. (1994) have reported that upon HIV-1 infection, thymic-purified TN and CD1a$^-$ TN cells readily produce viral particles into the culture supernatant, whereas purified CD1a$^-$ TN cells do not support viral entry. These findings suggest that in our DC population the presence of DC + T complexes might be a prerequisite for the detection of viral RNA in the TN thymocytes subset. Upon interacting with thymic DC, viral particles could replicate at low levels and/or be transmitted by cell-to-cell contact to associated thymocytes. It is interesting to note that even if viral replication is likely to occur in DC culture, no viral particles are detected in the supernatant. A recent study has suggested that in vitro-purified blood DC might internalize newly formed virions in a DC-T cell microenvironment (Cameron et al., 1994). A similar mechanism may occur in thymic DC, and thus newly formed virions from DC-associated thymocytes and/or from DC themselves might be rapidly internalized. These hypotheses could provide an explanation for the higher level of ISH signals observed in DC found in complexes compared to those in nonthymocyte-associated DC. Moreover it could explain the absence of viral particles in culture supernatants and could also account for the increased viral transmission observed with MT4 cocultured with 6- or 12-days postinfected DC compared to MT4 cocultured with 2-days postinfected DC.

Our results show that the presence of HIV-1 induces generalized profound morphological changes and DC killing. After 6 days of culture, only 50% of the HIV-1-infected DC remained viable compared to 80% in uninfected DC. In addition, half of these cells, compared to 5% among uninfected DC, exhibited abnormal vacuolization as observed in EM analysis. The addition of filtered supernatants collected from DC after 1 or 6 days of HIV-1 infection did not induce an increase of the cellular mortality in uninfected DC cultures. This result indicates that the induction of cell death observed in HIV-1-infected DC cultures is not mediated by soluble cellular- or viral-derived product(s) but is rather a consequence of DC-to-virus interaction(s).

Apoptosis is a form of cell death which is important in a wide range of physiological settings and is induced by diverse stimuli including HIV infection (Vaux, 1993; Reed, 1994; and review in Oyaizu and Pahwa, 1995). Apoptosis of in vitro-purified thymic DC could explain the mortality observed after exposure to HIV-1. However, as observed by EM, HIV-1-infected DC display ultrastructural characteristics which are typical of cell necrosis. Indeed, DC exhibit distension of mitochondria and organelles and an absence of nuclear fragmentation typical of apoptosis. HIV-1 virions could mediate DC toxicity through interaction with the plasma membrane. In this regard, sufficient level of virus interaction has been shown to result in increased cytoplasmic vacuolization (Fermin and Garry, 1992). Alternatively, DC killing may require the expression of viral gene(s). Additional studies will be necessary to understand the mechanism of HIV-mediated DC killing.

We observed that HIV-1 infection of DC in vitro induces the release of one or several heat-labile soluble factors in the culture supernatant which mediate the killing of thymocytes but not DC death. This product could be actively released into the medium as a response to the interaction of HIV with DC and/or passively released following the observed DC death. As demonstrated by ISH analysis of lymph nodes from HIV-1-infected individuals, HIV-1-mediated programmed cell death (PCD) occurs predominantly in bystander cells and not in productively infected cells (Finkel et al., 1995). Moreover, HIV infection of reconstituted thymus in SCID mice resulted in a rapid apoptosis of CD4$^+$ T cells (Aldrovandi et al., 1993; Bonyhadi et al., 1993). HIV-1 infection of thymic DC in vivo could lead to the release of a killing factor that in turn could mediate thymocyte PCD. As shown in our ISH analysis, a percentage of HLA-
DR^+)-infected cells likely express viral genes (Table 2 and Fig. 2). Thus, the release in the culture supernatant of viral gene products may promote PCD. HIV-1-mediated killing of thymocytes could alternatively implicate mechanisms involving release of cellular factors such as cytokines from infected DC. In this regard, expression of cytokines has been found to be increased either in patients with AIDS or in vitro after HIV-1 infection of primary cells including PBMCs, purified thymic, or blood monocytes (Borghi et al., 1990; Capobianchi et al., 1995). Up-regulated release of cytokines such as tumor necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β) has been proposed to program cells for death as a function of cell type, differentiation state, and the presence of other growth factors (Kekow et al., 1990; Axelrad, 1990; Meyaard et al., 1992; Bhalla et al., 1992). These products may in turn induce cell death either directly or indirectly by modulating the expression of other intermediates. More experiments are in progress to determine the identity of the factor(s), their mode of action, and the mechanism by which they are released from DC upon HIV infection.

Based on in vitro observations, thymic DC could be involved at different levels in the immune dysfunction in AIDS. Importantly, thymic DC could act in vivo as a reservoir for HIV-1 by nursing newly formed virions and preventing them from destruction by the immune system. This association can eventually lead to a cell-to-cell transmission of viral particles from DC to cells able to productively replicate virus. CD4+ precursor cells, such as CD4+CD8+ thymocytes were found to be highly susceptible to HIV-1 infection (De Rossi et al., 1990). Subsequent destruction of HIV-1-infected precursor cells might affect the replenishment of the CD4+ lymphocyte compartment in the peripheral blood. HIV infection of thymic DC in vivo could also, as observed in vitro, induce profound physiological changes and cell death in DC populations which could in turn affect their normal function in T cell selection in the thymus. Finally, HIV-1 infection of thymic DC may also participate in vivo in the reduction of mature T cells by releasing a soluble factor that could induce killing of thymocytes. The subsequent decrease in the rate of CD4+ cell replenishment may influence disease progression particularly in pediatric AIDS.

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