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Immunology

Three populations of cells with dendritic morphology exist in peripheral blood, only one of which is infectable with human immunodeficiency virus type 1

(infection/dendritic cell/precursor)

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ABSTRACT **Conflicting data have been reported with regard to the infectability, dysfunction, and depletion of dendritic cells (DCs) in human immunodeficiency virus (HIV) disease. These discrepancies could potentially be explained by the existence of multiple subsets of cells with dendritic morphology in peripheral blood. The isolation of DCs in humans is accomplished through negative selection until a morphologically pure population is obtained. Recently, DC precursors purified from peripheral blood by negative selection have been observed to develop into functionally and morphologically mature DCs. In this report we identify three populations of cells in peripheral blood that have or can develop a dendritic morphology. The first population, when allowed to mature in culture, develops a dendritic morphology and gains the expression of HB15, a marker of DCs in blood, thymus, skin, and lymphoid organs. The second population expresses HB15 and has the phenotypic and morphologic characteristics of mature DCs. The third population is morphologically very similar to mature DCs but does not share the same T-cell-stimulatory activity and is the only population that is infectable with HIV. Understanding the heterogeneity of cells of dendritic lineage and/or morphology in the peripheral blood will aid in understanding their role as antigen-presenting cells in general and as potential participants in the immunopathogenesis of HIV disease.**

Dendritic cells (DCs) are bone marrow-derived, antigen-presenting cells that are identified by their exceptional ability to present antigen to and activate T cells; a dendritic morphology; expression of high levels of major histocompatibility complex (MHC) class II and adhesion molecules; and the lack of B, T, natural killer (NK), and monocytic cell surface markers (reviewed in ref. 1). A recent report suggested a role for DCs in human immunodeficiency virus (HIV) infection in that HIV-pulsed DCs were demonstrated to infect superantigen-activated $CD4^+$ T cells (2). However, there are several conflicting reports in the literature with regard to infection, depletion, and dysfunction of DCs in HIV-infected individuals. HIV has been demonstrated to efficiently infect DCs in some (3–5) but not all studies (6, 7). Up to 21% of DCs from HIV-infected individuals were reported to be infected by *in situ* hybridization analysis (8) and were found to be depleted and dysfunctional in HIV disease (4, 8). In contrast, other studies have found that DCs from HIV-infected patients were neither infected (6, 9) nor depleted (6) and retained their ability to stimulate T cells in an autologous mixed lymphocyte reaction (MLR) (6).

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A number of methodologies have been described to isolate and purify mature DCs from peripheral blood mononuclear cells (PBMCs). A common denominator of these techniques is a duration of *in vitro* culture of 1–2 days in order for cells to mature (2–9). Certain methods of DC purification deplete T cells by sheep red blood cell (SRBC) rosetting and monocytes by adherence, prior to the culture period (2, 6, 10, 11). It has been suggested that it is difficult to identify cells with dendritic morphology in peripheral blood; however, a population of round cells could be identified that mature in culture into DCs as defined by surface antigen expression, morphology, and function (10, 11).

In the present study, we have identified three distinguishable populations of cells that have or can develop a dendritic morphology after purification. The first population is similar to the previously described DC precursors that are purified as round, MHC class II-positive cells that develop a dendritic morphology in culture. The second population appears to be fully mature DCs with characteristic morphology, phenotype, and function. They can be identified in PBMCs that have not undergone any *in vitro* culture or purification procedures and are indistinguishable from DCs that have been isolated after 1–2 days in culture. The third population copurifies with DCs that have been isolated after 1–2 days in culture; these cells have a dendritic morphology by light microscopy and express moderately high levels of MHC class II molecules but are inefficient stimulators of $CD4^+$ T cells in an autologous MLR. This third population is the only one that is infectable with HIV, even though all three populations express CD4.

METHODS

Medium and Reagents. RPMI 1640 (BioWhittaker) was supplemented with 2 mM glutamine (BioWhittaker) and 10% normal AB^+ human serum (Advanced Biotechnologies, Columbia, MD). Monoclonal antibodies (mAbs) to either HB15a (12), CD56 (Biosource International, Camarillo, CA), or CD16 (AMAC, Westbrook, ME) was incubated at 25 μg/ml with goat anti-mouse IgG beads (Dynal, Lake Success, NY) according to the instructions of the manufacturer. Fluorescein- or phycoerythrin-labeled anti-CD3, -CD14, -CD16, -CD19, and -HLA-DR (Becton Dickinson); fluorescein-labeled anti-

Abbreviations: DC, dendritic cell; $Fc_\gamma R$, Fc_γ receptor; HIV, human immunodeficiency virus; mAb, monoclonal antibody; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; NK, natural killer; PBMC, peripheral blood mononuclear cell; RT, reverse transcriptase; SRBC, sheep red blood cell.

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CD32 and anti-CD64 (Medarex, Lebanon, NH); fluorscein-labeled goat anti-mouse $F(ab')_2$ (Biosource International); biotinylated anti-HLA-DR (Becton Dickinson); and red670-labeled streptavidin (GIBCO/BRL) were used.

DC Purification. Populations of cells with dendritic morphology were obtained from PBMCs by the following procedures (Fig. 1). DC precursors were prepared as described (11), with the following modifications. After depletion of T cells by SRBC rosetting and depletion of monocytes by adherence to human Ig-coated plates, B lymphocytes were depleted on plates coated with goat anti-human IgG/A/M (100 μg/ml) (Tago) prior to flow cytometric purification of HLA-DR$^+$ and CD3$^-$, CD14$^-$, CD16$^-$, CD19$^-$, and CD56$^-$ cells. Alternatively, for experiments in which further cell surface marker analysis was performed, nonadherent cells were depleted by two incubations with magnetic beads specific for CD2, CD14, and CD19 (Dynal), CD16, and CD56 at a ratio of 40 beads per cell for 1 hr at 4°C.

Mature DCs were isolated by culturing PBMCs or SRBC/T-cell rosettes for 18–36 hr and layering over 12.5% (wt/vol) metrizamide (Sigma). After centrifugation at 650 × *g* for 10 min at 4°C, the buoyant cells were collected and depleted of T, B, NK, and monocytic cells as described above. In some experiments an initial step gradient of 9% metrizamide was used.

HB15a is a mAb that has been shown to bind DCs in peripheral blood (12). HB15$^+$ HLA-DR^{bright} cells were separated from the HB15$^-$ Fc_γ receptor type I-positive ($Fc_\gamma RI^+$) HLA-DR^{dim} population by staining with an anti-HLA-DR mAb and sorting according to brightness into bright and dim populations with a Coulter EPICS C flow cytometer.

Cell Staining. PBMCs were stained in three colors with HB15 and fluorescein-labeled goat anti-mouse $F(ab')_2$; phycoerythrin-labeled anti-CD3, -CD14, -CD16, -CD19, -CD20, and -CD56; and biotinylated anti-HLA-DR followed by red670-streptavidin and analyzed on a Coulter EPICS C. Purified cell populations (2×10^4) were centrifuged onto slides and stained with the Dako quick staining kit using alkaline phosphatase (Dako).

MLRs. B cells and CD4$^+$ T cells were isolated with anti-CD19 and anti-CD4 magnetic beads and monocytes were purified by adherence. During the overnight culture of the CD4$^+$ T-cell/magnetic bead conjugates, contaminating monocytes engulfed the beads and thus were efficiently depleted. Graded numbers of stimulators were mixed with CD4$^+$ T cells (10^5) in RPMI 1640 supplemented with 10% normal AB$^+$ human serum. On the third or fifth day of culture, 0.5 μCi (18.5 kBq) of [*methyl*-^{3}H]thymidine (New England Nuclear) was added and the cells were harvested 16 hr later [Tomtec (Orange, CT) automatic harvester] and incorporated radioactivity was counted (Betaplate 1205; Wallac, Gaithersburg, MD).

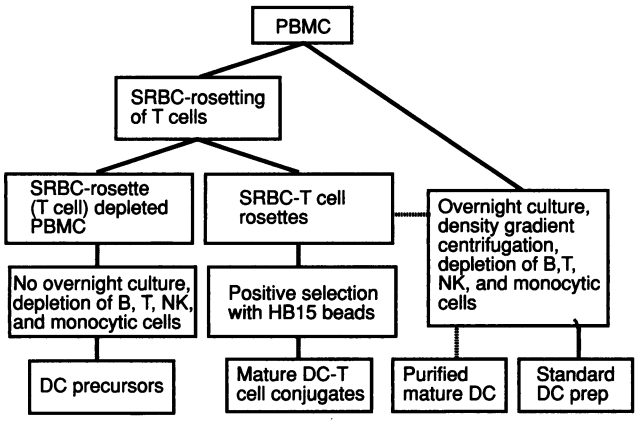

FIG. 1. Preparation of cell populations with dendritic morphology from PBMCs.

HIV Infection. Purified cell populations (10^5 cells per well) were treated with HIV_{IIIB} or HIV_{BaL} (Advanced Biotechnologies) at a multiplicity of infection of 10^{-4} or with the early-passage brain isolate HIV_{YU-2} (13) for 24 hr. Every 2–3 days supernatant was removed for measurement of reverse transcriptase (RT) activity (14). Cells were refed twice a week. PCR for HIV DNA, using SK38/39 primers, was performed 48 hr after infection, as described (15).

RESULTS

Study of DCs After *in Vitro* Culture Prior to Purification. DCs were isolated by standard techniques (4, 8) and contained <2% total contaminating B, T, NK, or monocytic cells by flow cytometry. Over 90% of the cells had a dendritic morphology consisting of lobulated nuclei and undulating dendritic processes. The DC preparation was doubly stained for HB15 and HLA-DR. Two populations were identified, one population was HB15$^+$ and HLA-DR^{bright} and the second population was HB15$^-$ and HLA-DR^{moderate} (Fig. 2*B*). When these populations were further analyzed, it was found that the HB15$^-$ population also stained dimly for CD32 ($Fc_\gamma RII$) (Fig. 2*C*) and CD64 ($Fc_\gamma RI$) (Fig. 2*D*), while the HB15$^+$ cells were negative for both $Fc_\gamma RI$ and $Fc_\gamma RII$ (Fig. 2 *C* and *D*).

Having identified two populations of cells with dendritic morphology in DC suspensions prepared by standard methods from peripheral blood, we then separated the two populations to >96% purity for further analysis. The HB15$^+$ population exhibited very weak staining for CD4 (Fig. 2*E*), whereas the

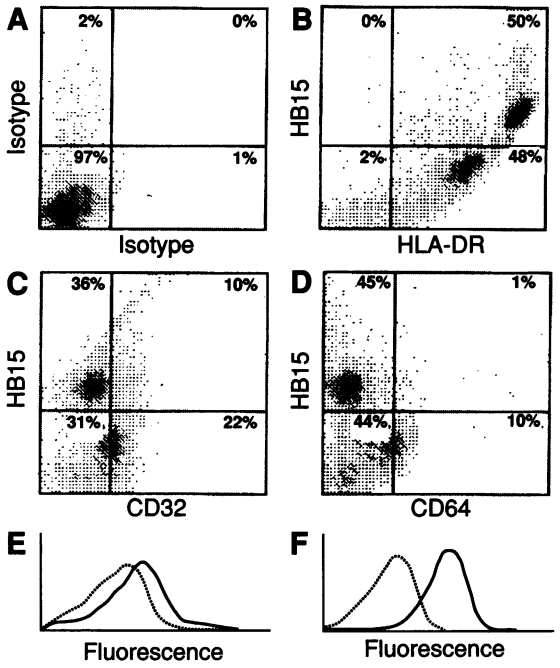

FIG. 2. Cytofluorometric analysis of cells with dendritic morphology obtained from peripheral blood. DC preparations were stained in two colors with control isotypes (*A*) and with antibodies to HB15 and HLA-DR (*B*), CD32 (*C*), and CD64 (*D*) and analyzed by cytofluorometry. The HB15$^+$ cells often had increased nonspecific staining with some mAbs as observed here with CD32 (*C*). When these cells were analyzed as a pure population no CD32 staining was observed. The HB15$^+$ (*E*) and HB15$^-$ $Fc_\gamma RI^+$ (*F*) cells with dendritic morphology were isolated and analyzed for CD4 expression. The expression of CD4 on the HB15$^+$ cells was very dim and often became negative if the cells remained in culture for extended periods of time. The figure illustrates data from one individual that were representative of data from five individuals studied.

$HB15^-$ $Fc_\gamma RI^+$ cells had a more pronounced expression of CD4 (Fig. 2*F*), similar to monocytes (data not shown). When the $HB15^-$ $Fc_\gamma RI^+$ cells were kept in culture for up to 5 days they did not develop HB15 or CD14 positivity (data not shown). The two populations were immunohistochemically stained for HLA-DR and HB15 (Fig. 3). Comparison of the two populations of cells by light microscopy, cytofluorometric analysis (Fig. 2), and immunohistochemical staining (Fig. 3) revealed that the two cell types had similar morphologic features but differed in their staining with anti-HB15. The use of light microscopy alone could not differentiate these two populations of cells.

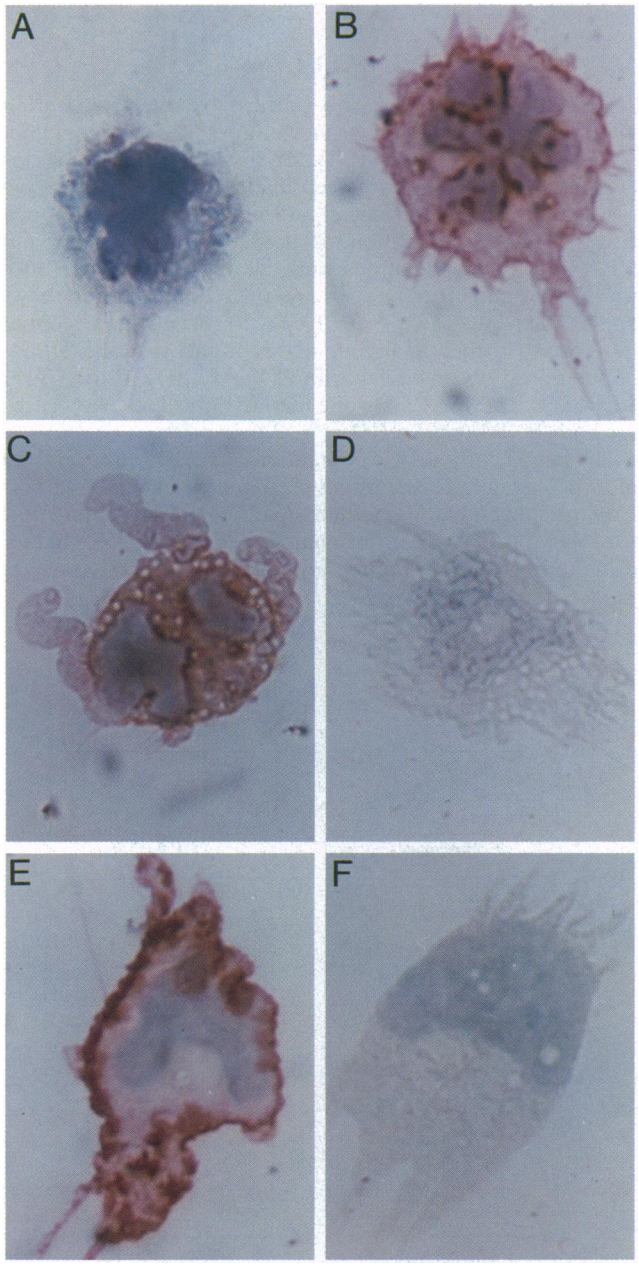

FIG. 3. Immunohistochemical staining of cells with dendritic morphology obtained from peripheral blood. HLA-DR^{bright} cells were stained with control isotype (*A*) or with antibodies to HLA-DR (*B*) and HB15 (*C*). $HB15^-$ $Fc_\gamma RI^+$ cells were stained with control isotype (*D*) or with antibodies to HLA-DR (*E*) and HB-15 (*F*). Because of the large numbers of cells required for the purification procedures, the presented cells were obtained from multiple donors and were stained at different times. In this regard, the $HB15^-$ $Fc_\gamma RI^+$ HLA-$DR^{moderate}$ cell (*E*) appears brighter than the HLA-DR^{bright} cell (*B*) on immunohistochemical staining with HLA-DR; this is due to the inherent variability in experimental conditions. Data represent selected cells from four individuals to demonstrate the diversity of morphologies observed. (×1000.)

Study of DCs Without *in Vitro* Culture Prior to Purification. The isolation of a population of round cells from PBMCs that expressed moderate levels of HLA-DR has been reported (10, 11). These cells, when allowed to mature in culture, develop into functional DCs. We have also identified a similar population of DC precursors in peripheral blood. They were $HB15^-$ and $CD4^+$ (Fig. 4 *A* and *B*) and, when allowed to mature, they demonstrated a dendritic morphology and expressed HB15 (Fig. 4*C*). Thus, DC precursors present in peripheral blood were able to mature into $HB15^+$ DCs.

Given recent reports on the difficulty in identifying mature DCs in peripheral blood without a prolonged culture period (10, 11), as well as the identification of precursors in peripheral blood which develop into DCs when left in culture, we attempted to determine whether DCs were actually present in the morphologically mature form in peripheral blood and could be detected without confounding purification procedures. Three-color cytofluorometric analysis was performed on freshly isolated PBMCs with the first color being a mixture of mAbs to T, B, NK, and monocytic cells. The cells that did

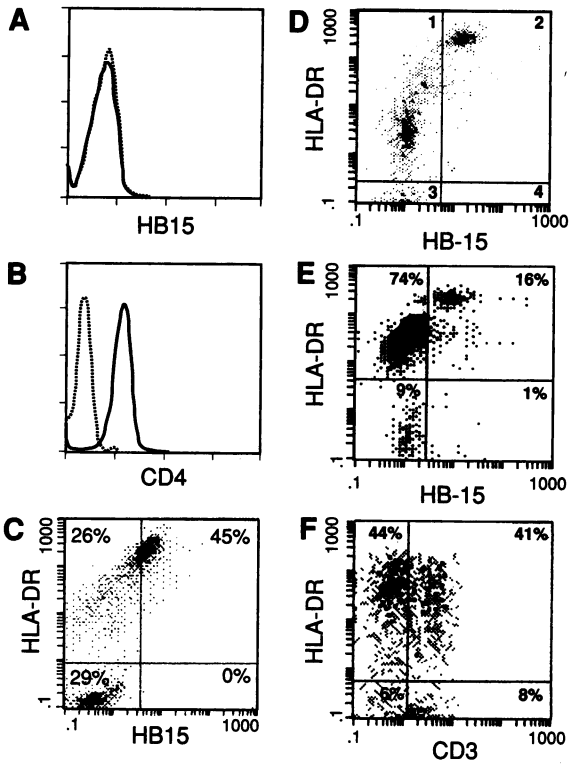

FIG. 4. Cytofluorometric analysis of DC populations in peripheral blood. DC precursors increase their HLA-DR expression and become $HB15^+$ when allowed to mature in culture. (*A* and *B*) Day 0 cells stained for HB15 (*A*) and CD4 (*B*). Dotted line is the isotype control. (*C*) HLA-DR and HB15 expression after 5 days in culture. Negative/positive gates for both colors were determined by using isotype controls with 98% of cells falling in the double negative gate as in Fig. 2*A*. (*D*) Three-color cytofluorometric analysis of freshly isolated PBMCs, without *in vitro* culture, demonstrates a population of $CD3^-$ $CD14^-$ $CD16^-$ $CD19^-$ $CD20^-$ $CD56^-$ (first color), $HB15^+$ HLA-DR^{bright} DCs. (*E*) HB15 and HLA-DR staining of SRBC/T-cell rosettes after overnight culture demonstrates $HB15^+$ DCs. (*F*) DCs isolated directly from PBMCs by use of HB15-specific magnetic beads demonstrate conjugates of DCs and T cells, HLA-DR^{bright} and $CD3^+$. The histograms shown are gated in such a way that free T cells that have fallen off the DCs are not shown. The HB15 beads did not bind purified T cells, suggesting that the T cells present copurified with the DCs. Data are representative of three other individuals tested.

not stain were further analyzed for the expression of HLA-DR and HB15. A population of very bright HLA-DR and HB15$^+$ cells could be identified; they accounted for 0.05–0.1% of the total PBMCs (Fig. 4*D*) and appeared morphologically identical to DC isolated by *in vitro* culture methods.

DC purification procedures that included depletion steps such as SRBC rosetting could not readily identify cells with dendritic morphology unless the resulting cell suspensions were cultured *in vitro*. Thus, we analyzed the cells that were removed by SRBC rosetting for the presence of mature DCs. When the SRBC/T-cell rosettes were cultured overnight and placed on a metrizamide gradient, the low-density cells contained a population of HB15$^+$ HLA-DR^{bright} cells (Fig. 4*E*). The HB15$^+$ cells that copurified with the SRBC-rosetted T cells represented 0.02–0.05% of the initial PBMCs and had the characteristic morphology of DCs on phase-contrast microscopy. To demonstrate that the DCs were bound to T cells, magnetic beads specific for HB15 were added to PBMCs, extensively washed, and allowed to detach from the cells overnight. Flow cytometric analysis revealed two populations of cells that were HLA-DR^{bright}, one of which was also CD3$^+$, suggesting a conjugate between DCs and T cells (Fig. 4*F*). Thus, since DCs readily bound to T cells (1) (Fig. 4*F*), it is highly likely that in separation procedures that utilized SRBC rosetting, the mature DCs clustered with the T cells and formed conjugates that were depleted. Likewise, in DC purification procedures that do not employ removal of T cells by SRBC rosetting and that utilize *in vitro* culture prior to purification, the resulting cells are likely a mixture of morphologically mature DCs present before *in vitro* culture and precursor-derived DCs that matured in culture, as well as a population of HB15$^-$ cells with dendritic morphology.

Functional Analysis of the Different Populations of Cells with DC Morphology. The hallmark of mature DCs is their exceptional ability to stimulate in an autologous MLR (1). HB15$^+$ cells were highly efficient stimulators of autologous CD4$^+$ T cells when compared with monocytes and the HB15$^-$ Fc$_\gamma$RI$^+$ cells with dendritic morphology (Fig. 5*A*).

Autologous MLR between mature HB15$^+$ DCs isolated from SRBC/T-cell rosettes and HB15$^+$ DCs obtained by cell sorting from DC precursors after 2 days of maturation demonstrated that the precursor-derived DCs were less efficient at stimulating CD4$^+$ T cells (Fig. 5*B*).

HIV Infectability of the Different Populations with Dendritic Morphology. The DC precursors (no *in vitro* culture), HB15$^+$, and HB15$^-$ Fc$_\gamma$RI$^+$ populations were exposed to HIV$_{IIIB}$, HIV$_{BaL}$, or the primary isolate HIV$_{YU-2}$ for 24 hr, washed, and monitored for infection by released RT activity (Fig. 6 *A* and *B*) or HIV DNA PCR (Fig. 6 *C–E*). Neither the HB15$^+$ cells nor the DC precursors produced RT activity or demonstrated *gag* DNA with any strain of virus used, whereas the HB15$^-$ Fc$_\gamma$RI$^+$ population demonstrated efficient infection.

DISCUSSION

We have analyzed three populations of cells obtained from PBMCs that do not stain with typical T, B, NK, or monocytic cell markers and that manifest dendritic morphology or the ability to develop such morphology. The first population has been described previously as DC precursors that are present in peripheral blood without overnight culture and can mature into DCs (10, 11). A second population had the characteristic morphology and T-cell-stimulatory function of DCs (1); they were HB15$^+$ and were identifiable in peripheral blood without *in vitro* culture. In this study, these DC precursors were HB15$^-$; however, they acquired HB15 positivity as they matured. A third population of cells demonstrated a dendritic morphology by phase-contrast, immunohistochemical, and electron microscopic examinations (data not shown); had moderate levels of

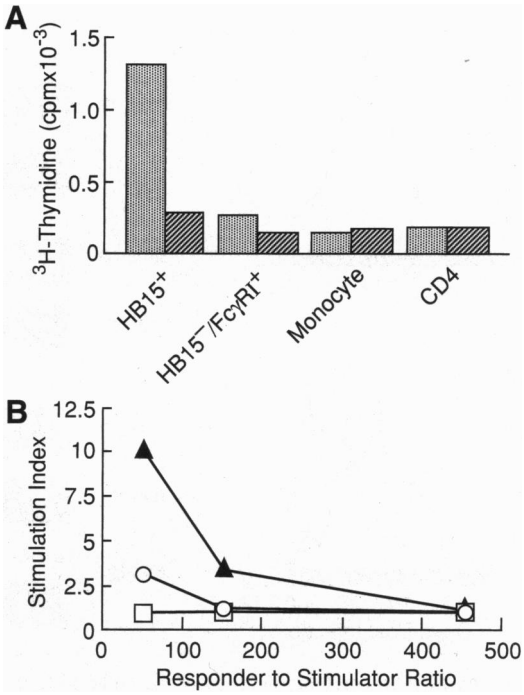

FIG. 5. Stimulatory capacity of cells with dendritic morphology in an autologous MLR. (*A*) HB15$^+$ DCs obtained by overnight culture are much more efficient in stimulating autologous CD4$^+$ T cells than monocytes or the Fc$_\gamma$RI$^+$ cells with dendritic morphology. ^{3}[H]Thymidine counts are from autologous cocultures of stimulators plus CD4$^+$ T cells; stimulator/responder ratio was 1:30 (stippled bars) or 1:100 (hatched bars). Cultures were pulsed for 16 hr on day 3 and then harvested. (*B*) When the HB15$^+$ DCs are separated into precursor-derived DCs and the morphologically mature DCs present in peripheral blood, the latter stimulate better in an autologous MLR. ▲, Mature HB15$^+$ DCs from peripheral blood; ○, HB15$^+$ DCs derived from DC precursors; □, monocytes. Stimulation index is calculated by dividing the [^{3}H]thymidine counts from the stimulator-plus-responder cell cultures by the counts from the responder cells alone. Cultures were run in triplicate and the standard error for each point was <25%. Data are representative of one out of eight individuals tested.

HLA-DR; were and remained CD14$^-$; expressed low levels of Fc$_\gamma$RI and Fc$_\gamma$RII; did not stimulate significantly in an autologous MLR; and were infectable by HIV *in vitro*.

DCs in peripheral blood, interdigitating DCs in lymph nodes, and Langerhans cells of the skin have been reported to express HB15 (12), and in this report DC precursors were demonstrated to become HB15$^+$ as they matured. It has been reported that CD34$^+$ progenitor cells that are cultured in the presence of granulocyte/macrophage-colony-stimulating factor and tumor necrosis factor α develop into CD1a$^+$ dendritic/Langerhans cells (16, 17). In preliminary experiments, we have observed that DCs derived from CD34$^+$ progenitors also express HB15 (unpublished observations). These findings suggest that HB15 may be a marker expressed by DCs which appears soon after their differentiation from progenitor cells and remains expressed on DCs in the skin, blood, and lymphoid organs.

The induction of "monocyte accessory cells" from CD14$^+$ monocytes treated with interleukin 4 has been described (18). These cells had a dendritic morphology, were CD14$^-$, and had an increased efficiency in presenting antigen to T cells as compared with untreated monocytes. Our HB15$^-$ Fc$_\gamma$RI$^+$ cells with dendritic morphology may be of similar derivation and, in fact, may be more closely related to monocytes.

Standard methods of isolating mature DCs from peripheral blood employ an *in vitro* culture period (2–9). Two populations of HB15$^+$ DCs were present after this culture period prior to further purification steps. The first population of HB15$^+$ DCs

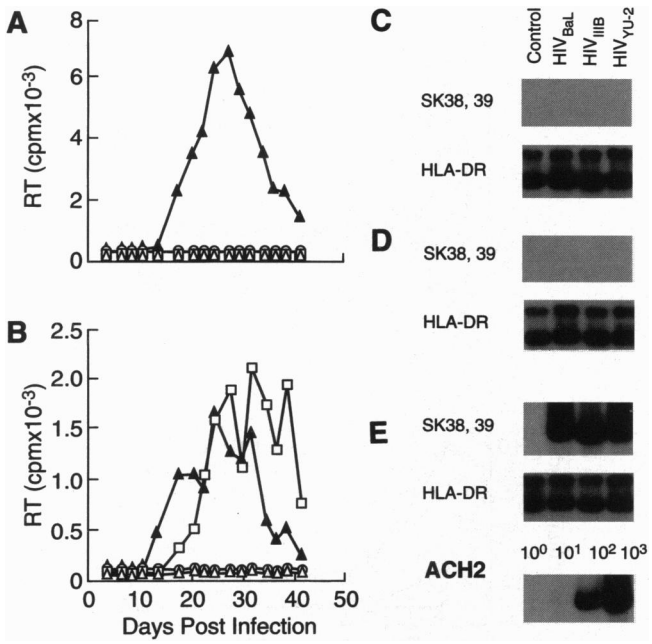

FIG. 6. *In vitro* infectability with HIV of cells with dendritic morphology obtained from peripheral blood. HB15$^-$ Fc$_\gamma$RI$^+$ cells are productively infected (as shown by RT activity) with HIV$_{IIIB}$ (*A*) and HIV$_{BaL}$ (*B*), whereas HB15$^+$ DCs and DC precursors are not infectable. ○, HB15$^+$ DCs; △, DC precursors without *in vitro* culture prior to infection; ▲, HB15$^-$ Fc$_\gamma$RI$^+$ cells; □, monocytes. Samples were run in duplicate cultures and tested for RT activity in duplicate assays. PCR for HIV DNA using *gag* (SK38/39) primers demonstrates that HB15$^+$ DCs obtained by overnight culture (*C*) and DC precursors with no *in vitro* culture (*D*) are not infectable with HIV, whereas the HB15$^-$ Fc$_\gamma$RI$^+$ cells (*E*) are efficiently infected with all three strains of virus tested. Each lane for SK38/39 primers contained the DNA from 10,000 cells, 2000 for HB15$^-$ Fc$_\gamma$RI$^+$. Each lane for HLA-DR primers contained DNA from 2000 cells. Data are from a single donor and are representative of two others. ACH2 cells, at the number of cells indicated, were run as a positive control for HIV-1 DNA copy number.

were derived from DC precursors that were originally HB15$^-$ and that matured and acquired HB15 positivity during the *in vitro* culture period. These DC precursors had most likely migrated from the bone marrow and were en route to various tissues to mature into DCs and Langerhans cells. They had intense HLA-DR staining in their perinuclear regions as shown by immunohistochemistry and pinocytotic vesicles as shown by electron microscopy (D.W. and A.S.F., unpublished observations), suggesting that they were engulfing antigens in order to express them on their surface in association with MHC class II molecules, a function ascribed to antigen-presenting cells (19). The second population was morphologically mature DCs that were present in peripheral blood without *in vitro* culture and were HB15$^+$ upon isolation. They were bound to T cells and were lost during cell fractionation procedures when SRBC rosetting was used. Functionally, they differed from the precursor-derived DCs in that they were more potent in the autologous MLR, suggesting a difference between *in vivo* and *in vitro* maturation of DCs.

The HB15$^+$ DCs that were already present in PBMCs or that were derived from DC precursors by *in vitro* culture were not infectable with HIV. The HB15$^-$ Fc$_\gamma$RI$^+$ cell population, which we submit is derived from monocytes and represents an alternative population with dendritic morphology in peripheral blood, was easily infectable with HIV. Analysis of the three populations of cells with dendritic morphology by microscopic methods was unable to differentiate the various populations. These observations may help to explain the varying results of DC infection, dysfunction, and depletion observed with HIV infection (2–9). In addition, they may lend to the further delineation of the diverse functions of cells with dendritic morphology, as well as a better understanding of the role of such cells in the pathogenesis of HIV disease.

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