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Interactions of CCR5 and CXCR4 with CD4 and gp120 in Human Blood Monocyte-Derived Dendritic Cells[1]

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Dendritic cells (DC) and macrophages play an important role in the generation of immune responses and transmission of HIV infection. It has been recently found that, in the presence of gp120, CD4 can be efficiently coimmunoprecipitated by anti-CXCR4 antibodies from lymphocytes and monocytes but not from blood monocyte-derived macrophages. The gp120–CD4–CXCR4 complex formation paralleled the ability for these cell types to support X4 (LAV) HIV-1 envelope glycoprotein (Env)-mediated fusion. Here we report that, unlike macrophages but similar to lymphocytes and monocytes, human blood monocyte-derived DC allow efficient complex formation among the HIV-1 coreceptor CXCR4, the primary receptor CD4, and the Env gp120 (LAV) which parallels their fusion ability with cells expressing HIV-1 Env (LAV). In addition, DC behaved similarly to macrophages, lymphocytes, and monocytes in their ability to support formation of complexes between CD4 and the other major HIV-1 coreceptor CCR5 even in the absence of gp120 as demonstrated by CD4 coimmunoprecipitation with anti-CCR5 antibodies. Further, the amount of gp120–CD4–CXCR4 (or CCR5) complexes was proportional to the extent of cell fusion mediated by the HIV-1 Env (LAV or JRFL, respectively). These results demonstrate that of all the major types of host cells important for HIV-1 infection, the first central stage in the entry mechanism, the formation of gp120–CD4–coreceptor complexes, is not impaired except for the formation of the gp120–CD4–CXCR4 complex in macrophages. Therefore, for most CD4+ target cells restraint(s) on productive HIV-1 infection appears to occur at stages of the virus life cycle subsequent to the gp120–CD4–coreceptor complex formation.

Key Words: HIV-1; CD4; CXCR4; CCR5; gp120; dendritic cells; membrane fusion.

INTRODUCTION

Dendritic cells (DC) are the most potent generators of primary and secondary immune responses. They are found in the dermis, in blood, and in lymphoid tissue, particularly in T-cell-rich regions, where they can efficiently deliver virus to CD4+ T cells. However, there has been some controversy regarding the ability of HIV-1 to productively infect DC although this can be due to the inherent heterogeneity of DC and the use of different subpopulations in different studies. It was initially found that the virus can replicate in DC (Patterson and Knight, 1987; Langhoff *et al.*, 1991) but a later report suggested that the replication in cultured DC is inefficient although infectious virus can be effectively transmitted

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from DC to CD4+ T cells (Cameron *et al.*, 1992). However, there is general agreement that DC can transmit infectious HIV-1 to CD4+ cells even in the absence of a productive infection in the DC (Cameron *et al.*, 1992; Pinchuk *et al.*, 1994; Pope *et al.*, 1994). After the discovery that the chemokine receptors CCR5 and CXCR4 serve as major HIV-1 coreceptors (reviewed in Dimitrov and Broder, 1997; Dimitrov, 1997; Dimitrov *et al.*, 1998), it was demonstrated that entry of R5 (BaL, JRFL, SF162, ADA) and X4 (IIIB) HIV-1 isolates in mature DC is efficient and mediated by CCR5 and CXCR4, respectively (Granelli-Piperno *et al.*, 1996).

It was proposed that the initial stages of HIV-1 entry into cells involve the formation of a trimolecular complex among the HIV-1 gp120 which is a part of its envelope glycoprotein (Env), the primary receptor CD4, and the coreceptors (Golding *et al.*, 1995; Dimitrov, 1996). By using a coimmunoprecipitation technique, the formation of such a complex was demonstrated in CD4+ T cell lines (Lapham *et al.*, 1996; Dimitrov *et al.*, 1998) and in primary peripheral blood CD4+ T cells, monocytes, and macrophages (Xiao *et al.*, 1999; Dimitrov *et al.*, 1999). However, formation of a complex among CXCR4, CD4, and gp120 from a T cell line adapted (TCLA) X4 HIV-1 strain (LAV) was found to be impaired in blood monocyte-derived macrophages (Dimitrov *et al.*, 1999). This finding suggested a plausible explanation for an observation that has been known for many years—that T cell line adapted (TCLA) X4 HIV-1 strains do not infect macrophages as efficiently as CD4+ T cells (reviewed in Dimitrov and Broder, 1997). It is clear that TCLA X4 HIV-1 Env cannot mediate efficient fusion in macrophages (Broder and Berger, 1995) and it is likely that viral entry is the major point of restriction to infection; however, infection could also be restricted at a postentry step (Schmidtmayerova *et al.*, 1998) in a manner similar to what was observed for mature DC.

Because DC share a common progenitor with monocyte-derived macrophages we hypothesized that blood monocyte-derived DC could exhibit an impaired interaction among CXCR4, CD4, and gp120 although they support entry by TCLA X4 HIV-1 strains. To test this hypothesis and to further characterize the interactions among coreceptors, CD4, and gp120, we investigated the formation of trimolecular complexes in human blood monocyte-derived DC. The results demonstrate that in the DC, as in the other major types of cells important for HIV-1 infection, the first main stage of the entry mechanism, the formation of gp120–CD4–coreceptor (CCR5 or CXCR4) complexes, is not impaired. Therefore, with the exception of macrophages, resistance to productive HIV-1 infection, stages of the virus life cycle subsequent to the gp120–CD4–coreceptor complex formation are affected.

MATERIALS AND METHODS

Cells, Vaccinia Viruses, gp120, sCD4, and Antibodies

Human monocytes were obtained from peripheral blood. Monocytes were allowed to differentiate to DC (over 85% CD1a+) as described previously (Rubbert *et al.*, 1998), and 3×10^7 of each cell type were used per sample. The HIV Env gp120 was produced by coinfection of BS-C-1 cells (ATCC CCL26) with vaccinia virus recombinant vPE6 via the hybrid vaccinia virus-T7 system (Fuerst *et al.*, 1986) with a multiplicity of infection of 10 PFU/cell under serum-free medium (OPTI-MEM, Life Tecnologies, Gaithersburg, MD) conditions and purified from the culture supernatants 30 h postinfection by affinity chromatography using lentil lectin–Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ) as previously described (Earl *et al.*, 1994). The anti-CD4 polyclonal antibody T4-4 was obtained through the AIDS Research and Reference Reagent Program from R. Sweet (SmithKline Beecham Pharmaceuticals). The anti-CCR5 mAb 5C7 was a kind gift from L. Wu. The anti-CXCR4 mAb 4G10 was raised against a peptide from the CXCR4 N-terminus.

Immunoprecipitation

DC (3×10^7 per sample) were washed once with phosphate-buffered saline (PBS) and then resuspended in PBS at a final density of 10^7/ml. Immunoprecipitating antibodies at the required concentration, typically 1.5–3 μg/ml, were added to the cell suspension and incubated with gentle mixing for 1 h at 37°C. Cells were then pelleted by centrifugation and resuspended in lysis buffer (1% Brij97, 5 mM iodoacetamide (added immediately before use), 150 mM NaCl, 20 mM Tris (pH 8.2), 20 mM EDTA, and protease inhibitors) at 4°C for 1 h with gentle mixing. The nuclei were pelleted by centrifugation at 14,000 rpm for 25 min in a refrigerated Eppendorf centrifuge. Protein G–Sepharose beads (Sigma, St. Louis, MO) prewashed with PBS were added to the samples and incubated at 4°C for 14 h. The beads were then washed four times with 1 ml of ice-cold lysis buffer. Samples were then eluted by adding 4× sample buffer for SDS–PAGE gel and boiled for 5 min. They were run on a 10% SDS–PAGE gel and were electrophoretically transferred to

nitrocellulose membranes. The membranes were blocked with 20 mM Tris–HCl (pH 7.6) buffer containing 140 mM NaCl, 0.1% Tween 20, and 5% nonfat powdered milk. These membranes were incubated with the respective antibodies and then washed, incubated with horseradish peroxidase-conjugated secondary antibodies, and developed by using the supersignal chemiluminescent substrate from Pierce (Rockford, Il).

Flow Cytometry

Cells (typically 0.5 min) were incubated for 1 h on ice with the antibodies (10 μg/ml) and then the cells were washed and incubated for another hour on ice with rabbit IgG (10 μg/ml) (Sigma) and then washed and incubated for 1 h with an anti-mouse phycoerythrin-conjugated polyclonal antibody (Sigma). The cells were washed and fixed with paraformaldehyde on ice for 10 min. The flow cytometry measurements were performed with a FACSCaliber (Becton–Dickinson, San Jose, CA). Calibrating beads with a known number of phycoerythrin molecules were purchased from Becton–Dickinson and used for calibration of the signal intensity. The number of receptor molecules was approximately estimated from the known signal intensity by using the equation describing the intensity of the calibrating beads assuming that each antibody molecule is conjugated on average with two phycoerythrin molecules and binds to two receptor molecules.

Cell Fusion Assay

The cell–cell fusion assay was previously described (Nussbaum *et al.*, 1994). The extent of fusion was quantitated colorimetrically.

RESULTS

Flow Cytometry Analysis of CD4, CCR5, and CXCR4 Concentrations at the DC Surface

We have shown previously that the ability to detect of CCR5–CD4 and CXCR4–CD4–gp120 complexes is dependent on the receptor surface concentration (Xiao *et al.*, 1999). When the surface concentration of CD4 or CCR5 (or CXCR4) is very low complexes can still be formed but they may not be detectable. Therefore we tested four independent sources of DC for the surface expression of the receptor molecules. We found that the surface concentrations varied widely from donor to donor (Fig. 1). There was no correlation among the cell surface concentrations of CD4, CCR5, or CXCR4 although individual 1 showed the highest CD4, CCR5, and CXCR4 concentrations. Compared to macrophages the levels of CD4 and CCR5 in DC were higher while CXCR4 was at lower surface concentrations. These results are consistent with previous reports (Zaitseva *et al.*, 1997; Lee *et al.*, 1999; Rubbert *et al.*, 1998) although quantitative comparison is difficult besause of donor to

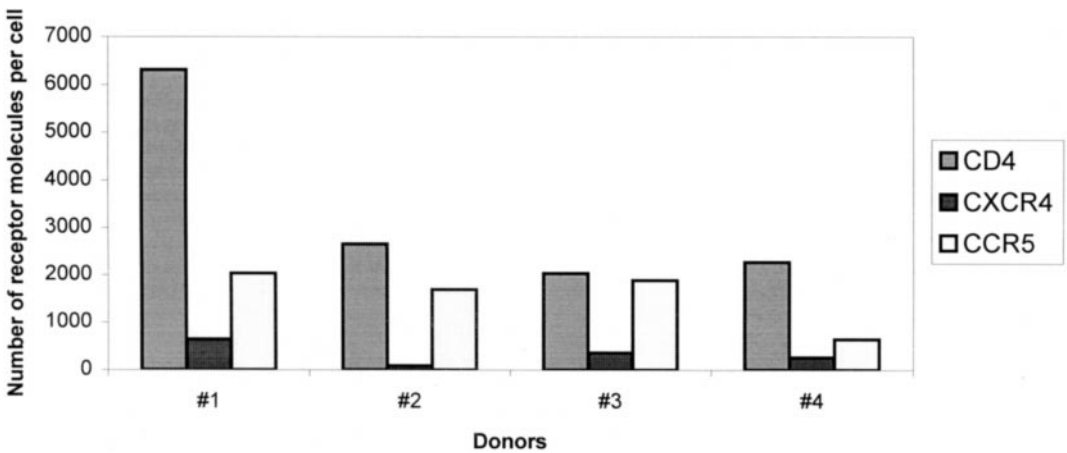

FIG. 1. Flow cytometry analysis of DC from four different donors (1–4) stained with the anti-CD4 mAb OKT4 (left), the anti-CXCR4 mAb 4G10 (middle), and the anti-CCR5 mAb m180 (right). The numbers on the vertical axis represent the number of PE molecules bound to the DC surface where the background is subtracted and are approximately equal to the number of receptor molecules at the cell surface.

donor variability, differences in culture conditions, and assay antibodies. We further extensively characterized DC from donors 1 and 2 because of their higher CD4 cell surface concentrations.

Fusion of DC with Cells Expressing R5 and X4 HIV-1 Env

Previously we and others have found a general correlation between the levels of surface expression of CD4 and CCR5 (or CXCR4) and the extent Env-mediated cell fusion (Kozak *et al.*, 1997; Zaitseva *et al.*, 1997; Dimitrov *et al.*, 1999). To determine whether such a correlation existed in DC we measured cell fusion using two assays: syncytium formation (Fig. 2) and β-galactosidase reporter-gene expression (Fig. 3). We found that for DC the extent of fusion was greater for cells expressing higher amounts of CD4 and CCR5; the fusion mediated by the R5 HIV-1 Env (JRFL) was more efficient than that mediated by the X4 HIV-1 Env (LAV). Importantly, and in contrast to macrophages which have relatively high surface CXCR4 concentration but do not support efficient fusion mediated by X4 Env (LAV), DC from all donors which expressed low levels of CXCR4 efficiently fused with cells expressing LAV Env (Fig. 3 and data not shown). Therefore, consistent with previously reported observations (Granelli-Piperno *et al.*, 1996), inefficient replication of HIV-1 in DC should be related to later stages of the virus life cycle.

Formation of Complexes between CD4 and CCR5 and among CD4, CXCR4, and gp120 in DC

We have previously shown that CD4 forms complexes with CCR5 even in the absence of any ligands including gp120 (Dimitrov *et al.*, 1998; Xiao *et al.*, 1999) and that gp120 can induce an association between CD4 and CXC4 (Lapham *et al.*, 1996; Dimitrov *et al.*, 1998) in a variety of cell lines and in primary cells including T cells, monocytes, and macrophages. However, despite the biological importance of DC for transmission of HIV-1, complex formation has not been measured at their surface. By using anti-CXCR4 and anti-CCR5 mAbs for immunoprecipitation of CXCR4 and CCR5, respectively, we found that, like primary T cells and monocytes, DC are able to support the formation of CD4–CCR5 and gp120–CD4–CXCR4 complexes (Fig. 4). These results demonstrate that DC, at least those derived from human blood monocytes, are not impaired in their ability to form gp120–receptor–coreceptor complexes further confirming the hypothesis that efficient formation of those complexes is required for efficient HIV-1 fusion (Broder and Dimitrov, 1996).

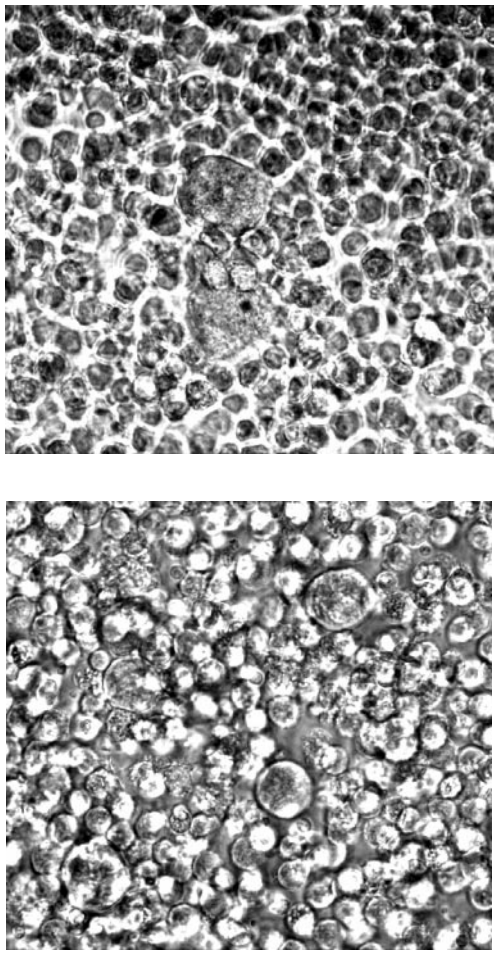

FIG. 2. Formation of syncytia as a measure of fusion between DC and cells expressing X4 (A) or R5 (B) Env. DC (10^5) were mixed in a 96-well plate with either TF228 (10^5) expressing the X4 LAV Env or U937-10 cells expressing the R5 JRFL Env after infection with recombinant vaccinia virus encoding the gene for the JRFL Env (vCB28). Pictures were taken 12h after mixing the cells.

DISCUSSION

This study is the first to demonstrate the existence of CD4–CCR5 and gp120–CD4–CCR5 complexes on the surface of mature human blood monocyte-derived DC, extending our previous observations in other HIV-1 target cell types. Together, these data demonstrate that the formation of complexes among CD4, gp120 of the Env, and CCR5 molecules occurs in all major types of cells which are biologically relevant for the transmission of HIV-1 infection. Interestingly, although the concentration of CXCR4 in DC is

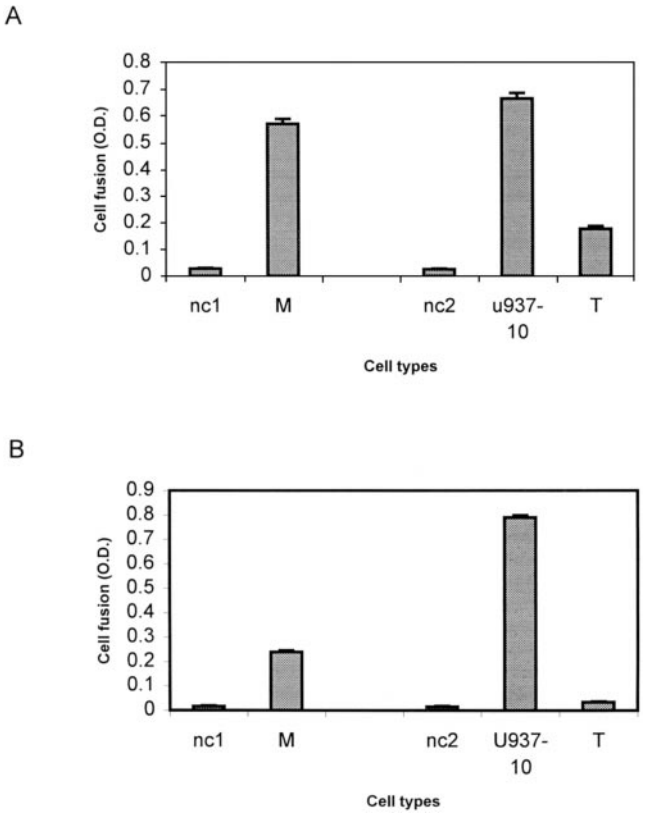

FIG. 3. Fusion between DC and cells expressing X4 (A) or R5 (B) Env as measured by the β-galactosidase assay previously described in Nussbaum *et al.* (1994). DC were infected with the recombinant vaccinia virus vTF7-3 (encoding T7 RNA polymerase) and incubated overnight (12 h) at 31°C. The DC were obtained from donors 1 (A) and 2 (B). TF228 cells expressing constitutively LAV Env (TF228) or U93710 infected with vaccinia viruses encoding an HIV-1 Env (JRFL) were used as effector cells. The effector cells, which were infected with a reporter virus vCB21R-LacZ encoding the *Escherichia coli* LacZ gene linked to the T7 promoter, were mixed with the DC at a ratio of 1:1 (total number of cells equaling 2×10^5 in 96-well plate format). Fusion was allowed to proceed for 4 h and quantitated by a colorimetric assay of β-galactosidase activity as optical density (OD) in detergent cell lysates. The experiments were performed in duplicates; the averages and deviations are shown. For comparison, fusion of U937 cells (subclone 10) with TF228 cells are shown (nc denotes negative control; M, M-tropic Env (JRFL), and T, T tropic Env (LAV)).

much lower than in macrophages with comparable CD4 concentrations, the mature DC support the formation of complexes and efficient cell fusion mediated by an X4 Env while macrophages do not (Dimitrov *et al.*, 1999). This suggests that the restriction in the ability of macrophages to support cell fusion mediated by a TCLA Env is related to some unique property(s) of the macrophages which has yet to be fully elucidated.

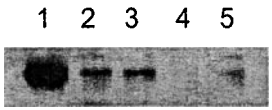

FIG. 4. Formation of complexes between CD4 and CCR5 or CXCR4 in presence of gp120. Lane 1 is an example of CD4 Western blot after CD4–CCR5 coimmunoprecipitation by the anti-CCR5 mAb 5C7 from 8×10^6 3T3.CD4.CCR5 cells. Lanes 2 and 3 represent CD4 Western blots of DC (3×10^7 cells) lysates immunoprecipitated by the anti-CCR5 mAb 5C7 in the absence (lane 2) or presence (lane 3) of JRFL gp120 (10 μg/ml). Lanes 4 and 5 represent CD4 Western blots of DC (3×10^7 cells) lysates immunoprecipitated by the anti-CXCR4 mAb 4G10 in the absence (lane 4) or presence (lane 5) of LAV gp120.

The formation of gp120–CD4–CCR5 (CXCR4) complexes could not only play an important role in the association of infectious HIV-1 to DC, but also might contribute to, or facilitate, the efficient transmission to CD4 T cells (Cameron *et al.*, 1992; Pope *et al.*, 1994). Although physical association of infectious virus with DC may not involved a specific binding of the Env to CD4 and coreceptor molecules, one could speculate, for example, that the crosslinking of CD4 with coreceptors by gp120 could lead to signal transduction and expression of molecules which may enhance the transmission of infectious virus. In this regard, it has recently been proposed that the efficient transmission of HIV-1 from antigen-presenting cells including DC is a major cause of the ongoing virus dissemination even under highly active antiretroviral therapy (Grossman *et al.*, 1999). In light of these new propositions the ability of the DC, the most potent antigen-presenting cells, to support efficient Env–CD4–coreceptor complex formation deserves further investigation not only for the development of drugs designed to disrupt these complexes but also for the development and application of vaccines derived from them.

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