INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) infection is characterized by the progressive depletion of CD4\(^+\) and CD8\(^+\) T lymphocytes, especially via apoptosis or programmed cell death (Ameisen & Capron, 1991). The HIV-1 envelope gp120 interacts with the CD4 molecule and several chemokine receptors, mainly CXCR4 and CCR5 (Berger et al., 1999). The differential phenotype displayed by CCR5- and CXCR4-using viruses, R5 and X4 viruses, respectively, appears to modulate HIV pathogenesis, and X4 isolates are usually associated with a faster decline in T cell counts and faster acquired immunodeficiency syndrome (AIDS) progression (Fauci, 1996).

CD8\(^+\) T cell apoptosis is observed in the peripheral blood isolated from HIV-infected subjects and could result from several mechanisms. CD8\(^+\) T cells are activated cells with increased cell surface expression of Fas, CD38 and tumour necrosis factor receptor 2 (TNFR2) (de Oliveira Pinto et al., 2002a; Herbein et al., 1998a; Mueller et al., 2001). Besides the Fas–Fas ligand (FasL) death signalling pathway (de Oliveira Pinto et al., 2002a; Herbein et al., 1998a; Mueller et al., 2001; Tateyama et al., 2000), tumour necrosis factor (TNF)-\(\alpha\)-TNFR2 interaction has been reported to trigger activation-induced T cell apoptosis of mature CD8\(^+\) T cells and cytotoxic T lymphocytes in animal models, especially in the presence of macrophages (M\(\phi\)) (Alexander-Miller et al., 1998; de Oliveira Pinto et al., 2002a; Herbein et al., 1998a; Vlahakis et al., 2001; Zheng et al., 1995). During the course of HIV infection, both uninfected and infected CD4\(^+\) T lymphocytes undergo apoptosis. In vitro, evidence of a direct killing of HIV-infected CD4\(^+\) T cells has been reported using reporter virus systems (Gandhi et al., 1998; Herbein et al., 1998b). In vivo, antigen-presenting cells have been found to trigger apoptosis of the bystander uninfected CD4\(^+\) T cells in the lymph nodes of HIV-infected individuals (Badley et al., 1997; Finkel et al., 1995; Herbein et al., 1998b; Oyaizu et al., 1997). M\(\phi\)-mediated apoptosis of CD4\(^+\) T cells is mediated via Fas–FasL interaction following CD4 cross-linking (Badley et al., 1997; Banda et al., 1992; Oyaizu et al., 1997). In addition to pro-apoptotic cytokines and viral proteins, CXCR4 participates in several HIV envelope pathogenic mechanisms that contribute to CD4\(^+\) T cell depletion such as syncytium formation (Berndt et al., 1998; Blanco et al., 2000; Vlahakis et al., 2001; Westendorp et al., 1995).

CXCR4, in addition to its role as a co-receptor for X4 HIV strains, modulates trafficking of immune cells (Feng et al., 1996; Loetscher et al., 1994). The interaction of the naturally occurring CXCR4 agonist stromal cell-derived factor-1\(x\) (SDF-1\(x\)) activates different signalling pathways that result in increased intracellular calcium levels (Bleul et al., 1996; Davis et al., 1997). These signalling events lead to a chemotactic response in T and B lymphocytes (Bleul et al., 1996), the stimulation of pre-B lymphocytes (Nagasawa et al., 1994, 1996), foetal and adult T cell development (Ara et al., 2003), T cell activation (Nanki & Lipsky, 2000) and
increased inflammatory response (Matthys et al., 2001). Low-molecular-mass molecules, such as the bicyclam AMD3100 [the octahydrochlorodihydrate salt of phenylbis-(methylen)-bis-(1,4,8,11-tetraazacyclotetradecane), recognize the same region of CXCR4 used by both dual- and T cell-tropic HIV-1 strains for virus entry and show a strong inhibitory activity against X4 HIV-1 isolates (de Clercq & Schols, 2001; Donzella et al., 1998; Doranz et al., 1997). AMD3100 inhibits the intracellular calcium signalling and chemotactic response elicited by the natural CXCR4 ligand SDF-1 in different cell types (Donzella et al., 1998).

To study the role of CXCR4 in HIV-mediated T cell apoptosis, we blocked the CXCR4 receptor using the small-molecule CXCR4 inhibitor AMD3100. CXCR4 blockade resulted in inhibition of CD8+ T cell apoptosis and, to a lesser extent, CD4+ T cell apoptosis, in peripheral blood mononuclear cells (PBMCs) isolated from HIV-infected subjects. Moreover, our results indicate that several factors could play a role in CXCR4-mediated T cell apoptosis in HIV infection: CD4/CD8 phenotype, viral envelope phenotype, T cell activation and T cell–Mφ intercellular contacts.

**METHODS**

**Clinical subjects.** This study enrolled a cohort of 44 patients aged 36–73 years with viral load ranging from less than 50 to more than 750,000 copies HIV RNA ml⁻¹, CD4 counts ranging from 125 to 776 (mm blood)⁻¹ and who were antiretroviral drug-naïve (n=6) or under highly active antiretroviral therapy (HAART) (n=38). Samples of peripheral blood were collected between January 1999 and June 2003 from volunteers attending clinics in Galveston (UTMB) or Besançon (France). The appropriate local Institutional Review Boards and Ethics Committees approved the study.

**Reagents.** AMD3100 was obtained from G. Henson (AnorMED) as dry powder and stored as a 1 mg ml⁻¹ water solution. Phycoerythrin (PE)-labelled anti-human CXCR4 mouse IgG2a monoclonal antibody (12G5 clone) (Becton Dickinson Pharmingen) was used to measure cell surface expression of CXCR4 on PBMCs.

**Viruses.** Primary virus isolates were recovered from the PBMCs of HIV-infected subjects and propagated in phytohaemagglutinin-stimulated PBMCs as described previously (Castro et al., 1988). All of these primary isolates were phenotyped using the MT2 assay (Koot et al., 1992). Aliquots (1 ml) were frozen at −70 °C.

**T lymphocyte counts.** The CD4 and CD8 cell counts were measured using flow cytometry (CellQuest; Becton Dickinson).

**Isolation and culture of peripheral blood lymphocytes (PBLs), PBMCs and primary Mφ.** Human PBLs and purified PBLs were prepared from peripheral blood of HIV-infected subjects as described previously (Herbein et al., 1998a, b). PBLs and PBMCs were cultivated in RPMI medium with 10% (v/v) foetal bovine serum in the presence and absence of AMD3100 (0, 10, 100 and 1000 ng ml⁻¹) for 18 h. To block PBL–Mφ intercellular contacts, the cell populations were separated by a semi-permeable membrane in six-well plates (0.4 μm pore size, Transwell; Costar). Adherent tissue culture-differentiated Mφ (>94% CD14+ by flow cytometric analysis) were cultured in RPMI medium supplemented with 10% (v/v) pooled AB human serum (Sigma) (Herbein et al., 1998a).

**Measurement of apoptosis.** The detection of apoptosis by terminal transferase dUTP nick end labelling (TUNEL) assay was performed as described previously and analysed by flow cytometry (Herbein et al., 1998b). To measure apoptosis by caspase assay, 1×10⁶ cells per sample were washed in PBS and then in Perm Wash buffer (Becton Dickinson Pharmingen). Cells were treated with 500 μl Cytofix-cytoperm solution (Becton Dickinson Pharmingen) for 20 min at 4°C. After one wash in Perm Wash buffer, cells were incubated for 1 h at room temperature with FITC-coupled anti-caspase 3 monoclonal antibody (Becton Dickinson Pharmingen). As a negative control, cells were incubated with a FITC-labelled isotype-matched mouse IgG1 antibody. After two washes in Perm Wash buffer, cells were resuspended in PBS and analysed by flow cytometry.

**Flow cytometry analysis.** For the TUNEL assay, caspase 3 assay or for CD95, CD38, HLA-DR, CD69 and TNFR2 detection, cells were analysed with a FACScan flow cytometer (Becton Dickinson) as reported previously (Herbein et al., 1998a). Briefly, PBMCs were gated on the basis of SSC and FSC and identified following CD45/CD14 labelling (Simultest Leucogate; Becton Dickinson). CD4 and CD8 were detected using peridinin chlorophyll protein (PerCP)-labelled anti-human CD4 and anti-human CD8 mouse IgG1 monoclonal antibodies, respectively (Becton Dickinson). For the detection of CD38, CD95, HLA-DR, TNFR2 and CD69 expression, 5×10⁵ cells were cultivated in the presence or absence of AMD3100 for 18 h. Cells were then collected, washed in 100 μl final volume PBS and stained with PerCP-labelled anti-human CD8 or anti-human CD4 (Becton Dickinson) mouse IgG1 monoclonal antibodies and with PE-coupled anti-human HLA-DR (Diaclone), CD38 (Becton Dickinson) and TNFR2 (R&D Systems) mouse IgG1 monoclonal antibodies, FITC-coupled anti-human CD95 and CD69 (Becton Dickinson) mouse IgG1 monoclonal antibodies. Isotypic controls were PerCP-, PE- or FITC-coupled mouse IgG1 antibodies. Data from 5×10⁴ cells were collected, stored and analysed by using CELLQUEST software (Becton Dickinson).

**Detection of chemokine levels.** RANTES (regulated-on-activation normal T-expressed and secreted) and MIP-1α (macrophage inflammatory protein) levels were determined using a commercial ELISA according to the manufacturer’s instructions (Quantikine human RANTES or MIP-1α immunoassay; R&D Systems).

**Statistical analysis.** All measurements are presented as means and standard deviation with statistical comparisons made between conditions using Student’s t-test for paired observations. The level of significance was set at P<0.05.

**RESULTS**

In PBMCs isolated from HIV-infected subjects, CXCR4-mediated apoptosis accounts for CD8+ T cell apoptosis and to a lesser extent for CD4+ T cell apoptosis

To examine the effect of CXCR4 blockade on spontaneous CD4+ and CD8+ T cell apoptosis, PBMCs isolated from HIV-infected subjects were treated for 18 h with increasing concentrations of AMD3100 (0, 10, 100 and 1000 ng ml⁻¹) and the rates of spontaneous CD4+ and CD8+ T cell apoptosis were measured by TUNEL assay. AMD3100 significantly decreased CD8+ T cell apoptosis, and to a lesser extent CD4+ T cell apoptosis, in a dose-dependent manner (P<0.05) (Fig. 1a, b). Similar results were obtained using a caspase 3 assay for the measurement of apoptosis (data not shown). In PBMCs isolated from the peripheral blood of HIV-negative subjects, the rates of CD8+ and
CD4⁺ T cell apoptosis were 2.1% (±0.8%) and 1.8% (±0.6%), respectively, in the absence of AMD3100 treatment, and following AMD3100 treatment modification of the rates was not statistically significant (data not shown).

CXCR4-mediated T cell apoptosis depends partially on the syncytium-inducing/non-syncytium-inducing envelope phenotype

We observed that the rates of CD8⁺ and CD4⁺ T cell apoptosis were 2.6-fold and 1.5-fold higher, respectively, in PBMCs isolated from AIDS subjects versus asymptomatic HIV-infected subjects (P<0.05) (Fig. 2a). AMD3100 inhibited 75% and 50% of CD8⁺ T cell apoptosis in PBMCs isolated from AIDS subjects and asymptomatic HIV-positive persons, respectively (P<0.01). By contrast, AMD3100 inhibited only 35% and 30% of CD4⁺ T cell apoptosis in PBMCs isolated from AIDS subjects and from asymptomatic HIV-positive persons, respectively (P<0.05) (Fig. 2a). Since syncytium-inducing (SI) strains emerge during AIDS in half of the patients, we determined the SI/non-syncytium-inducing (NSI) phenotype parallel to the rates of T cell apoptosis. Subjects infected with SI strains displayed higher rates of CD8⁺ and CD4⁺ T cell apoptosis than subjects infected with NSI strains (P<0.05) (Fig. 2b). Interestingly, AMD3100 significantly inhibited CD8⁺ T cell apoptosis, but inhibited CD4⁺ T cell apoptosis via CXCR4 in HIV infection
apoptosis to a lesser extent, in PBMCs isolated from subjects infected with SI strains, but also with NSI strains ($P<0.05$) (Fig. 2b).

In contrast to CXCR4-mediated CD4$^+$ T cell apoptosis, CXCR4-mediated CD8$^+$ T cell apoptosis depends on T cell activation

Since T cell activation results in apoptosis, we measured CD4$^+$ and CD8$^+$ T cell activation as determined by CD38, CD95, HLA-DR, CD69 and TNFR2 expression in PBMCs isolated from HIV-infected persons and left untreated or treated with AMD3100. In contrast to HLA-DR, TNFR2 and CD69 expression, AMD3100 decreased both CD38 and CD95 expression on the CD8$^+$ T cell surface by 52% and 35%, respectively ($P<0.05$ and $P<0.05$, respectively) (Fig. 3a, b). By contrast, we did not observe a significant decrease in cell surface activation markers on CD4$^+$ T cells following AMD3100 treatment (Fig. 3a, b). Blockade of CD8$^+$ T cell apoptosis, but not of CD4$^+$ T cell apoptosis, correlated positively with the inhibition of T cell activation following AMD3100 treatment ($r^2=0.72$; data not shown).

CXCR4-mediated T cell apoptosis, especially CXCR4-mediated CD8$^+$ T cell apoptosis, is increased in the presence of Mφ

Since Mφ trigger T cell apoptosis, we assessed whether CXCR4 is involved in Mφ-mediated T cell apoptosis in peripheral blood isolated from HIV-infected persons. Therefore, we measured CD4$^+$ and CD8$^+$ T cell apoptosis in PBMCs versus autologous PBLs. Rates of CD4$^+$ and CD8$^+$ T cell apoptosis were increased by three- to sixfold in PBMC cultures versus PBL cultures (Fig. 4a, b). Separation of Mφ from PBLs by a semi-permeable membrane blocked both CD8$^+$ and CD4$^+$ T cell apoptosis, indicating that intercellular contact between Mφ and either CD8$^+$ or CD4$^+$ T cells is necessary for apoptosis induction (data not shown). Both CD8$^+$ and CD4$^+$ T cell apoptosis correlated positively with Mφ-to-PBL ratio ($r^2=0.67$ and

![Fig. 3. Role of T cell activation on CXCR4-mediated T cell apoptosis in HIV-infected persons. The dot plots (a) and histograms (b) show the cell surface expression of CD38, CD95, CD69, HLA-DR and TNFR2 on CD8$^+$ and CD4$^+$ T cells in PBMCs isolated from HIV-infected persons and left untreated or treated for 18 h with AMD3100 at 1000 ng ml$^{-1}$. For the dot plots, the percentages of cells present in the quadrants are shown. For the histograms, the results are mean values (±SD) of five independent experiments. Solid bars, untreated; open bars, AMD3100; *, $P<0.05$.](image-url)
$r^2 = 0.55$, respectively) (Fig. 5). AMD3100 blocked, in a dose-dependent manner, CD8$^+$ T cell apoptosis and to a lesser extent CD4$^+$ T cell apoptosis in PBMC cultures, but not in autologous PBLs, isolated from HIV-infected persons and treated with increasing concentrations of AMD3100 (100, 1000 ng ml$^{-1}$) for 18 h. Results represent combined data from 10 independent experiments.

Even though numerous studies have investigated the effects

DISCUSSION

Our results demonstrate that several factors participate in CXCR4-mediated T cell apoptosis in HIV-infected persons: CD4/CD8 phenotype, SI/NSI envelope phenotype, T cell activation and T cell–Mϕ intercellular contacts.

Mϕ-to-PBL ratio and was more efficient for CD8$^+$ than for CD4$^+$ T cells (Fig. 5).

Fig. 4. Comparison of PBMCs versus autologous PBLs for CXCR4-mediated T cell apoptosis in HIV-infected persons. (a) Spontaneous CD8$^+$ T cell apoptosis was measured by TUNEL assay in PBMCs and autologous PBLs isolated from HIV-infected persons and left untreated or treated with increasing concentrations of AMD3100 (100, 1000 ng ml$^{-1}$) for 18 h. Results are representative data from 10 independent experiments. (b) Decreased spontaneous CD8$^+$ T cell apoptosis, and to a lesser extent CD4$^+$ T cell apoptosis, in PBMCs, but not in autologous PBLs, isolated from HIV-infected persons and treated with increasing concentrations of AMD3100 (black bars, untreated; hatched bars, 10 ng ml$^{-1}$; grey bars, 100 ng ml$^{-1}$; white bars, 1000 ng ml$^{-1}$) for 18 h. CD8$^+$ and CD4$^+$ T cells were gated and studied for apoptosis using TUNEL assay. Results represent mean values (±sd) of 10 independent experiments.

Fig. 5. Role of autologous Mϕ in CXCR4-mediated T cell apoptosis in HIV-infected persons. Apoptosis was measured by TUNEL assay using two-colour flow cytometric analysis on gated CD8$^+$ and CD4$^+$ T cells in PBLs cultivated in the presence of increasing concentrations of autologous monocyte-derived Mϕ and left untreated or treated with AMD3100 at 1000 ng ml$^{-1}$ for 18 h. Results represent combined data from independent experiments.
of HIV infection on T lymphocyte apoptosis, the factors involved in CXCR4-mediated T lymphocyte apoptosis in HIV-infected persons have not been reported so far. In vitro studies have shown previously that HIV isolates differ in their ability to induce cell death in CD4+ and CD8+ T cells (Blanco et al., 2001). Although the chemokine receptor CXCR4 is involved in both CD8+ and CD4+ T cell apoptosis (Berndt et al., 1998; Herbein et al., 1998a), our data show that, in the peripheral blood isolated from HIV-infected persons, CXCR4-mediated T cell apoptosis is triggered mostly in CD8+ T lymphocytes and to a lesser extent in CD4+ T lymphocytes. Several hypotheses might account for the reduced involvement of CXCR4 in apoptosis of CD4+ versus CD8+ T cells in HIV-infected persons. First, apoptosis of uninfected CD4+ T cells has been described to result from CD4 cross-linking by the HIV envelope and/or via CCR5 involvement (Algeciras-Schimnich et al., 2002; Banda et al., 1992; Oyaizu et al., 1997) and therefore might be less susceptible to CXCR4 blockade. Moreover, apoptosis of infected cells might account for CD4+ T cell apoptosis in HIV-infected patients (Gandhi et al., 1998; Herbein et al., 1998b). CD4+ T cell apoptosis increases proportionally with plasma viral load, which is a valid predictor of HIV-related disease (Saag et al., 1996), and a dynamic interaction between the replication of HIV-1 and the destruction of CD4+ T lymphocytes has been reported (Ho et al., 1995; Wei et al., 1995). In agreement with these data, we observed that the highest rates of CD4+ T cell apoptosis were measured when virus replication was not controlled, with viral load above 10 000 copies of HIV RNA ml−1 (data not shown). In this case, AMD3100 might only have a limited antiretroviral effect due to the presence of R5 HIV-1 strains, as a result of the multiple co-receptor usage of primary HIV-1 strains and the inability of AMD3100 to interfere with cell surface chemokine receptors other than CXCR4 (Doranz et al., 1997; Glushakova et al., 1998; Hatse et al., 2002). In agreement with this latter observation, the binding of the anti-CXCR4 monoclonal antibody 12G5 to the cell surface of PBLs was inhibited by AMD3100 in a dose-dependent manner (data not shown). Secondly, the anti-apoptotic effect of AMD3100 has been reported to be cell type-dependent (Blanco et al., 2000), suggesting that the blockade of apoptosis in CD8+ and CD4+ T cells might vary. This could be due to the fact that the conformation of the CXCR4 chemokine co-receptor may vary between CD8+ and CD4+ T lymphocytes, and/or that the density of CXCR4 molecules on the surface of CD4+ and CD8+ T cells may be different (Baribaud et al., 2001; Blanco et al., 2000; Labrosse et al., 1998; Lapham et al., 2002).

In HIV-infected persons, the chronic activation of the immune system, particularly in the CD8+ T cell subset, is probably one of the major causes of T cell death (Badley et al., 1999; Caggiari et al., 2000; de Oliveira Pinto et al., 2002b; Dryhol-Riise et al., 2001; Giorgi et al., 1999; Tilling et al., 2002). We observed that CD8+ T cell apoptosis did not directly result from increased virus replication in the peripheral blood, as measured by plasma viral load (data not shown), suggesting that non-viral factors, such as CD8+ T cell activation, might play a critical role in CD8+ T cell apoptosis in HIV-infected persons. In agreement with these data, we observed that, in contrast to CD4+ T cells, high levels of activation markers, such as CD38 and Fas, were expressed on the cell surface of CD8+ T cells from HIV-infected persons and were down-regulated following CXCR4 blockade. Interestingly, HIV-1 infection is associated with mucosal inflammation characterized by increased expression of CXCR4 (Olsnson et al., 2000), and CXCR4 stimulation has been reported recently to trigger T cell activation (Nanki & Lipsky, 2000). Thus, our data indicate that CXCR4 blockade by AMD3100 reduces CD8+ T cell activation and thereby could inhibit CD8+ T cell apoptosis in HIV-infected persons. Also, CXCR4 blockade could result in the inhibition of T cell chemotaxis and therefore could diminish CD8+ T cell–Mφ interactions that have been reported to trigger CD8+ T cell apoptosis (Herbein et al., 1998a; Vlahakis et al., 2001). In agreement with this hypothesis, we observed a decreased production of RANTES and MIP-1α in culture supernatants of PBMCs isolated from HIV-infected subjects and treated with AMD3100 (data not shown). Although T cell activation seems to be less involved in CD4+ T cell apoptosis than in CD8+ T cell apoptosis, we cannot rule out the possibility that CXCR4-mediated activation plays a role in CD4+ T cell death (Roggero et al., 2001).

Both Mφ-mediated CD4+ and CD8+ T cell apoptosis have been extensively described in HIV infection (Badley et al., 1997; Herbein et al., 1998a; Oyaizu et al., 1997). Mφ-mediated CD4+ T cell apoptosis has been reported to result from CD4 cross-linking and Fas–FasL interaction (Banda et al., 1992; Oyaizu et al., 1997), whereas Mφ-mediated CD8+ T cell apoptosis has been reported to involve CXCR4–gp120 interaction (Herbein et al., 1998a; Vlahakis et al., 2001). In agreement with these data, our results indicate that, in HIV-infected persons, Mφ-mediated CD8+ T cell apoptosis, and to a much lesser extent Mφ-mediated CD4+ T cell apoptosis, are CXCR4-dependent.

In conclusion, our study shows that several virus and immune factors play a role in CXCR4-mediated T cell apoptosis in HIV-infected persons. A better understanding of the factors involved in T cell apoptosis during HIV infection could lead to new therapeutic approaches that combine an antiretroviral regimen and immune reconstitution and might clear the reservoirs of virions in HIV-infected persons.

ACKNOWLEDGEMENTS

We thank the American Foundation for AIDS Research (02631-26-RGI) and the Agence Nationale de Recherche sur le SIDA (to G. H.) for financial support. We thank Patrick Hervé and Pierre Tiberghien (EFS, Besançon, France) for material assistance.
REFERENCES


Tilling, R., Kinloch, S., Goh, L. E. & 8 other authors (2002). Parallel decline of CD8+/-CD38+/- T cells and viremia in response to quadruple highly active antiretroviral therapy in primary HIV infection. AIDS 16, 589–596.


