INTRODUCTION

Human immunodeficiency virus (HIV-1) infection suppresses bone marrow hematopoiesis and causes various peripheral blood cytopenias (Harbol et al., 1994; Fauci, 1996; Jenkins et al., 1998; Koka et al., 1998; Moses et al., 1998). Thrombocytopenia (TP) is the most common form of cytopenia in HIV-1-infected patients and occurs in ~30% of cases (Patnaik, 1989; Harbol et al., 1994). HIV-associated TP (platelet count < 100,000 mm$^{-3}$ in the absence of opportunistic infections, neoplasms, and antiretroviral drug therapy) is a multifactorial disorder involving immune destruction of platelets in the periphery and reduced thrombopoiesis in the bone marrow (Walsh et al., 1984; Ratner, 1989; Ballem et al., 1992; Zauli et al., 1996). HIV-1 has been implicated in the reduction of bone marrow thrombopoiesis by several studies. HIV-1 transcripts have been detected in megakaryocytes of TP patients (Zucker-Franklin and Cao, 1989; Louache et al., 1991) and ineffective thrombopoiesis has been reported in these patients (Zauli et al., 1991; Ballem et al., 1992; Cole et al., 1998). In addition, megakaryocyte morphology has been reported to be abnormal in TP patients (Zucker-Franklin et al., 1989) and zidovudine treatment has been shown to improve platelet counts in these patients (Oksenhendler et al., 1989; Boyar and Beall, 1991). Most importantly, it has been suggested that HIV-1 infection of bone marrow megakaryocytes may contribute to the development of thrombocytopenia (Louache et al., 1991; Monte et al., 1992; Chelucci et al., 1998). However, conflicting results have been reported on the ability of HIV-1 to infect primary megakaryocytes and their precursors (Louache et al., 1992; Zauli et al., 1992; De Luca et al., 1993; Zauli and Davis, 1993), questioning the hypothesis that HIV-1 infection of bone marrow megakaryocytes may be one of the contributing factors in the development of thrombocytopenia.

In this study we aimed to determine whether HIV-1 from the bone marrow of TP and non-TP patients could infect primary megakaryocytes in vitro. We analyzed HIV-1 from the bone marrow because it has been shown that the HIV-1 quasispecies varies in different cellular compartments (Donaldson et al., 1994; Delwart et al., 1998; Poss et al., 1995; van't Wout et al., 1998; Voulgaropoulou et al., 1999). Recombinant HIV-1 strains were constructed using the hypervariable loop 3 (V3) of HIV-1 envelope from the bone marrow of TP and non-TP patients (Voulgaropoulou et al., 1999). We showed that HIV-1 strains from the bone marrow of TP patients were more pathogenic in vitro than HIV-1 strains from the bone marrow of non-TP controls (Voulgaropoulou et al., 1999). Specifically, four of six HIV-1 strains from the bone marrow of TP patients replicated in T-cell lines or primary macrophages, whereas HIV-1 strains from the bone marrow of non-TP patients did not replicate in T-cell lines and only one of five strains replicated in primary macrophages (Table 1) (Voulgaropoulou et al., 1999). In this report we identified the chemokine receptor molecules utilized by bone marrow HIV-1 for entry into susceptible cells and the chemokine receptor molecules expressed on CD34$^+$-cell-derived megakaryocytes. Finally, we showed that both X4 and R5 HIV-1 strains productively infected primary megakaryocytes in vitro.
TABLE 1

<table>
<thead>
<tr>
<th>Molecular clone</th>
<th>Tropism</th>
<th>CXCR4</th>
<th>GHOST-CCR5</th>
<th>GHOST-CCR3</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/BM9</td>
<td>T</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B/BM1</td>
<td>PBMC</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B/BM4</td>
<td>PBMC</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B/BM8</td>
<td>T</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>C/BM6</td>
<td>M</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<tr>
<td>C/BM11</td>
<td>M</td>
<td>-</td>
<td>+</td>
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<tr>
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<tr>
<td>J/BM2</td>
<td>-</td>
<td>-</td>
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<td>+</td>
</tr>
<tr>
<td>J/BM11</td>
<td>PBMC</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
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<tr>
<td>p120</td>
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<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>p125</td>
<td>M</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

* HIV-1 molecular clones from TP patients include A/BM9, B/BM1, B/BM4, B/BM8, C/BM6, and C/BM11. BM designates bone marrow HIV-1 strains and Bld designates blood HIV-1 strains. p120 and p125 are T-cell and macrophage tropic laboratory adapted HIV-1 molecular clones (Voulgaropoulou et al., 1999).

HIV-1 molecular clones capable of replication in primary macrophages or T-cell lines are designated as M or T, respectively. HIV-1 molecular clones capable of replication in peripheral blood mononuclear cells only are designated as PBMC. Replication competent molecular clones incapable of replication in PBMC, macrophages, or T-cell lines are designated by dashes (Voulgaropoulou et al., 1999).

Dashes represent background levels of RT activity (3 x 10^2 to 5 x 10^2 cpm/ml) and + represents levels of RT activity above background.

RESULTS

Coreceptor utilization of bone marrow HIV-1 molecular clones

This study includes 6 HIV-1-infected patients. Patients A, B, and C were diagnosed with HIV-associated TP, Patient D was diagnosed with nocardia infection in the bone marrow, and Patients J and K were diagnosed with Hodgkin's disease (Voulgaropoulou et al., 1999). Bone marrow aspirates and blood were obtained after informed consent, and DNA sequences encoding the V3 loop of HIV-1 envelope were amplified by nested polymerase chain reaction (Voulgaropoulou et al., 1999). Eleven HIV-1 recombinant clones were constructed from the bone marrow of three TP (A, B, and C) and three non-TP patients (D, J, and K), and two recombinant clones were constructed from the blood of two patients (J and K) (Table 1) (Voulgaropoulou et al., 1999). To identify the chemokine receptors utilized by the recombinant HIV-1 clones for entry into susceptible cells, GHOST cells expressing CXCR4, CCR5, and CCR3 chemokine receptors were infected with the molecular clones listed in Table 1. None of the molecular clones was able to replicate in GHOST parental cells, except p120, which replicated to low levels due to the presence of endogenous CXCR4 on the surface of these cells (data not shown). All bone marrow and blood isolates were able to replicate in GHOST–CCR5 cells (R5) regardless of their ability to replicate in primary macrophages (Table 1). The T-cell tropic isolates A/BM9 and B/BM8 were able to replicate in GHOST–CXCR4 cells (X4), whereas the T-cell tropic isolate J/Bld47 did not replicate in these cells, suggesting that J/Bld47 was unable to utilize CXCR4 for entry into GHOST cells (Table 1). Finally, one TP (A/BM9) and one non-TP (K/BM15) isolate replicated in GHOST–CCR3 cells (Table 1). The reference T-cell tropic molecular clone p120 (Voulgaropoulou et al., 1999) was able to replicate in GHOST–CXCR4 cells very efficiently but less efficiently in GHOST–CCR5 and -CCR3 cells, suggesting that p120 can utilize mainly CXCR4 but also CCR5 and CCR3 to enter susceptible cells (Table 1). The reference macrophage tropic molecular clone p125 (Voulgaropoulou et al., 1999) was able to replicate in GHOST–CCR5 cells very efficiently but less efficiently in GHOST–CXCR4 cells, suggesting that p125 can utilize mainly CCR5 but also CXCR4 for entry into susceptible cells (Table 1). None of the molecular clones was able to replicate in GHOST cells expressing CCR1, CCR2b, CCR8, BOB, BONZO, and V28 chemokine receptors except p120, which was able to replicate in GHOST–CCR1 and -CCR2b cells (data not shown). Therefore the bone marrow of TP patients is characterized by the presence of both X4 and R5 HIV-1 strains whereas, only R5 HIV-1 strains were present in the bone marrow of non-TP patients.

Chemokine receptor profile of CD34+-cell-derived megakaryocytes

CD34+ hematopoietic progenitor cells were grown in serum-free media supplemented with 50 μg/ml thrombopoietin (Tpo) for 7 days. Under these conditions, CD34+ hematopoietic progenitor cells have been shown to differentiate into CD41+ megakaryocytes (Zucker-Franklin and Kaushansky, 1996; Birkmann et al., 1997; Zauli et al., 1997; Schipper et al., 1998) (Fig. 1). Furthermore, CD34+-cell-derived megakaryocytes have been shown to be positive for CD4, the receptor for HIV-1 (Gewirtz et al., 1992; Kouri et al., 1993; Zauli et al., 1995; Basch et al., 1996; Lee et al., 1999) (Fig. 1). To determine the chemokine receptor profile of primary megakaryocytes, CD34+-cell-derived megakaryocytes were labeled by immunofluorescence using CXCR4-, CCR5-, and CCR3-specific monoclonal antibodies. Confocal microscopy determined that the chemokine receptors CXCR4, CCR5, and CCR3 were present in 100% of CD34+-cell-derived megakaryocytes (Fig. 1). Therefore primary megakaryocytes express CD4, CXCR4, CCR5, and...
CCR3 and may be susceptible to infection by both X4 and R5 HIV-1 strains. Furthermore, HIV-1 strains from the bone marrow of TP patients infected megakaryocytes to similar levels as HIV-1 strains from the bone marrow and blood of non-TP patients and the laboratory-adapted molecular clones p120 and p125. Viral release from HIV-1-infected megakaryocytes was measured to be ~190 pg of p24 per milliliter of culture supernatant, for all molecular clones studied (data not shown). Furthermore, the p24 value was similar for all time points tested during the 13-day infection (Days 3, 6, 9, and 13) for all molecular clones, suggesting that HIV-1 was constantly released from the infected megakaryocytes into the supernatant (data not shown). These results demonstrate that CD41+ megakaryocytes were productively infected by both X4 and R5 HIV-1 isolates in vitro.

**DISCUSSION**

The primary determinant of HIV-1 pathogenesis is the entry of HIV-1 into susceptible cells. HIV-1 entry is facilitated through the interaction of the HIV-1 envelope glycoprotein on the surface of the virion with the HIV-1 receptor molecules on the surface of target cells (Cairns and D’Souza, 1998; Lee et al., 1998). The HIV-1 cellular receptors have been identified as the differentiation marker CD4 (Dalgleish et al., 1984; Klatzmann et al., 1984; Maddon et al., 1986) and the chemokine receptors CXCR4 and CCR5 (Deng et al., 1996; Dragic et al., 1996; Feng et al., 1996). Other chemokine receptor molecules have also been shown to function as HIV-1 coreceptors in vitro (Choe et al., 1996; Doranz et al., 1996; Deng et al., 1997; Frade et al., 1997; He et al., 1997; Liao et al., 1997; Loetscher et al., 1997; McKnight et al., 1997; Rucker et al., 1997; Horuk et al., 1998; Jinno et al., 1998) but the biological significance of these molecules in vivo has not been established. The available evidence suggests that CXCR4 and CCR5 are the relevant chemokine receptors in vivo (Huang and Carmichael, 1996; Samson et al., 1996; Zhang et al., 1996; Smith et al., 1997; Stewart et al., 1997; Michael et al., 1998; Mummidi et al., 1998; Zhang and Moore, 1999) and are utilized by most or all HIV-1 isolates (Rucker et al., 1997; Xiao et al., 1998). In addition to CXCR4 and CCR5, the chemokine receptor CCR3 has been suggested to play a role in HIV-1 neuro tropism (He et al., 1997; Shieh et al., 1998; Zhang et al., 1998). Generally, CXCR4 mediates entry of T-cell tropic, syncytia-inducing (SI) isolates into CD4+ cells, whereas CCR5 mediates entry of macrophage tropic, non-syncytia, inducing (NSI) isolates into these cells (Alkhatib et al., 1996; Deng et al., 1996; Dragic et al., 1996; Feng et al., 1996; Speck et al., 1997).

In the present study, we aimed to determine whether HIV-1 in the bone marrow of TP patients can infect primary megakaryocytes in vitro, which may contribute to the development of thrombocytopenia. We studied two

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**FIG. 1.** Antibody staining of CD34+ hematopoietic progenitor cells grown in Tpo media for 7 days. The mouse monoclonal antibodies used were CD41, CD4, CXCR4, CCR5, and CCR3 (1–2 μg of antibody per 10^6 cells). IgG1 and IgG2a (1–2 μg of antibody per 10^6 cells) were used as isotype-matched negative control antibodies. Cells were stained with anti-mouse IgG conjugated to PE (1:100 dilution).
groups of patients, patients with TP (A, B, and C) and non-TP patients with hematopoietic dysfunction caused by the invasion of bone marrow with neoplasms or opportunistic pathogens (D, J, and K). We constructed recombinant viruses using the V3 loop of HIV-1 envelope from the bone marrow and blood of these patients (Voulgaropoulou et al., 1999) and determined the chemokine receptor molecules utilized by the bone marrow and blood HIV-1 strains and the chemokine receptor molecules expressed on megakaryocytes. We showed that all bone marrow and blood HIV-1 isolates utilize CCR5 for entry into susceptible cells, irrespective of their ability to replicate in primary macrophages. The use of CCR5 by most HIV-1 primary isolates has been reported by others (Simmons et al., 1996; Clapham and Weiss, 1997; Cairns and D’Souza, 1998) as well as the inability of several R5 HIV-1 strains to infect macrophages (Cheng-Mayer et al., 1997; Dittmar et al., 1997; Smyth et al., 1998; Hung et al., 1999). The T-cell tropic HIV-1 strains, characteristic of TP patients, utilized CXCR4 for entry into susceptible cells, whereas one TP and one non-TP HIV-1 strain utilized CCR3. One notable exception was the T-cell tropic blood isolate J/Bld47, which was able to replicate in MT-2 cells but not in Jurkat and Hut78 T-cell lines (Voulgaropoulou et al., 1999) and not in GHOST–CXCR4 cells (Table 1). The ability of X4 HIV-1 isolates to infect some T-cell lines but not others has been reported by others (Cheng-Mayer et al., 1988; Connor et al., 1993; Dumitrescu et al., 1994). Finally, we did not observe a correlation between CCR3 utilization and thrombocytopenia because CCR3 utilizing HIV-1 strains were isolated from both TP and non-TP patients. Therefore chemokine receptor analysis revealed that X4 HIV-1 strains were present only in the bone marrow of TP patients, whereas R5 HIV-1 strains were present in the bone marrow of both TP and non-TP patients.

To determine the susceptibility of megakaryocytes to HIV-1 infection, we analyzed the chemokine receptor

![FIG. 2. HIV-1 infection of CD34⁺-cell-derived megakaryocytes. HIV-1-infected megakaryocytes were detected by two-color immunofluorescence using CD41 monoclonal antibody (1–2 μg/10⁶ cells) and pooled human sera from HIV-1-infected patients (1:100 dilution). Uninfected cells stained with IgG1 isotype-matched antibody and human sera from HIV-1-infected patients served as negative control. The mouse monoclonal antibody CD41 was labeled with anti-mouse IgG conjugated to PE and HIV-1 antibodies were labeled with anti-human IgG conjugated to FITC (1:100 dilution).](image-url)
profile of megakaryocytes using confocal microscopy. Megakaryocytes were obtained from CD34+ hematopoietic progenitor cells grown in serum-free medium supplemented with Tpo. Under these conditions, CD34+ progenitor cells differentiate into CD41+ megakaryocytes after several days in culture (Zucker-Franklin and Kaushansky, 1996; Birkmann et al., 1997; Zauli et al., 1997; Schipper et al., 1998). Most importantly, these CD34+ cell-derived megakaryocytes have been reported to be morphologically identical to bone marrow megakaryocytes and to produce platelets in vitro that are indistinguishable from normal platelets (Choi et al., 1995a,b; Cramer et al., 1997). We showed, in agreement with published reports, that CD41+ megakaryocytes express surface CD4, CXCR4, and CCR5 (Gewirtz et al., 1992; Kouri et al., 1993; Zauli et al., 1995; Basch et al., 1996; Wang et al., 1998; Kowalska et al., 1999; Lee et al., 1999; Riviere et al., 1999) and may be susceptible to infection by both X4 and R5 HIV-1 strains. In addition, we report for the first time, the presence of the CCR3 chemokine receptor on CD34+-cell-derived megakaryocytes. Our results contradict the findings of Chelucci et al., who did not detect CCR3 mRNA on cells of the megakaryocytic lineage (Chelucci et al., 1999). Furthermore, Chelucci et al. (1999) did not detect CCR5 on the surface of megakaryocytes, an observation that contradicts our results and the results of others (Lee et al., 1999).

To determine whether primary megakaryocytes were susceptible to infection by HIV-1, we exposed CD34+ cell-derived megakaryocytes to HIV-1 from the bone marrow of TP and non-TP patients. We showed that CD41+ megakaryocytes were productively infected by both X4 and R5 HIV-1 strains. Our results contradict previous reports on the susceptibility of megakaryocytes to T-cell tropic but not macrophage tropic HIV-1 (Chelucci et al., 1998, 1999; Lee et al., 1999). Furthermore, HIV-1 strains from the bone marrow of TP and non-TP patients infected megakaryocytes to similar levels. Because we did not observe a difference in the ability of HIV-1 from TP or non-TP patients to infect megakaryocytes, we believe that the viral determinants of HIV-associated TP may lie outside the V3 loop of HIV-1 envelope. Alternatively, HIV-1 may cause TP indirectly as has been suggested by others (Molina et al., 1990; Louache et al., 1992; Zauli et al., 1992b,c).

In conclusion, we showed that the bone marrow of TP patients is characterized by the presence of both X4 and R5 HIV-1 strains, whereas only R5 HIV-1 strains were present in the bone marrow of non-TP patients. HIV-1 from the bone marrow of TP and non-TP patients infected megakaryocytes to similar levels, suggesting that the viral determinants HIV-associated TP may lie outside the V3 loop of the HIV-1 envelope. Furthermore, CD34+ cell-derived megakaryocytes express CD4, CXCR4, and CCR5 and are productively infected by both X4 and R5 HIV-1 strains. Finally, we showed that CD34+ cell-derived megakaryocytes express the chemokine receptor CCR3.

MATERIALS AND METHODS

Infection of GHOST cells with recombinant HIV-1

The GHOST HIV indicator cells [GHOST (3) parental, CXCR4, Hi-CCR5, and CCR3 (AIDS Research and Reference Reagent Program)] were used to determine the coreceptor molecules utilized by the HIV-1 recombinant clones for entry into susceptible cells. Briefly, 5 X 10⁶ cells were infected with ~400 ng of HIV-1 p24 gag overnight in the presence of Polybrene (20 µg/ml). The next day the cells were washed, fed with fresh media and maintained for 6 additional days, assaying for reverse transcriptase activity (RT) every 3 days. GHOST (3) cell lines express uniformly high levels of CD4 and low levels of endogenous CXCR4 (AIDS Research and Reference Reagent Program).

CD34+ cell isolation

CD34+ cells were mobilized with granulocyte-colony stimulating factor and/or granulocyte macrophage-colony stimulating factor from healthy volunteers after informed consent. Leukapheresis products were red cell depleted and CD34+ hematopoietic progenitor cells were isolated using the MACS CD34 progenitor cell-isolation kit, according to the manufacturer’s instructions (Miltenyi Biotec). Briefly, 2 X 10⁶ cells were magnetically labeled using hapten-conjugated CD34 monoclonal antibody (clone QBEND/10) and an anti-hapten antibody coupled to MACS MicroBeads. CD34-labeled cells were positively selected by passing them through MACS separation columns twice, to achieve 95% purity, and frozen in 90% fetal calf serum ([FCS] (Gibco, BRL]) and 10% DMSO (Sigma) until needed.

Confocal microscopy

Approximately 1 X 10⁶ cells were centrifuged on to Superfrost Plus microscope slides (Fisher) using a StatSpin Cytofuge (StatSpin, Inc.). Cells were fixed in 100% acetone or 4% paraformaldehyde for 10 min and incubated in PBS/BSA/Tween 20 buffer [1 X phosphate-buffered saline (PBS)/3% bovine serum albumin (BSA)/0.2% Tween 20] before the addition of antibodies. All antibody dilutions were made in PBS/BSA/Tween 20 buffer. Primary antibodies were incubated with cells for 1 h at room temperature and secondary antibodies were kept on cells for 30 min at room temperature. Excess antibody was removed by washing in PBS/BSA/Tween 20 buffer for 10 min by gentle agitation. The mouse monoclonal antibodies used were anti-human -CD41 (clone PM6/248, Chemicon International Inc.), -CD4 (clone QS4120, Calbiochem-Novabiochem Corp.), -CCR5 (clones 2D7 and 5C7, AIDS Research and Reference
Reagent Program), -CXCRI4 (clone 44708.111, R&D Systems, Inc.), and -CRI3 (clone 7B11, AIDS Research and Reference Reagent Program). Isotype controls were performed using purified mouse IgG1 and IgG2a (Calbiochem). Pooled human sera from 10 HIV-1-infected patients were used as a source for antibodies against HIV-1 (the generous gift of Dr. Max Arens), which recognized HIV-1 p24 and gp120 as detected by immunoprecipitation of metabolically labeled virus and by Western blot analysis of patient sera (data not shown). Mouse monoclonal antibodies were labeled with anti-mouse IgG conjugated to phycoerythrin (PE) (Boehringer Mannheim) and HIV-1 antibodies were labeled using anti-human IgG conjugated to fluorescein isothiocyanate (FITC) (Calbiochem-Novabiochem Corp.). Slides were mounted using 1% n-propyl gallate solution (50 mM EDTA/50% glycerol/100 mM Tris pH 8.0) and antibody staining was detected by Laser Scanning Confocal microscopy (Zeiss Axiovert with Biorad confocal scanning imaging system).

Infection of megakaryocytes with recombinant HIV-1

CD34+ hematopoietic progenitor cells were cultured in Tpo media: StemPro-34 SFM serum-free medium (Gibco, BRL) supplemented with 50 μg/ml thrombopoietin (Tpo) (R&D Systems Inc.), 2 mM L-glutamine (Bio-whittaker), and 100 μg/ml penicillin and streptomycin (BioWhittaker). At Day 7, 1 × 10⁵ cells were infected with ~400 ng of HIV-1 p24⁴⁰⁰ overnight. The next day, the cells were washed extensively and cultured in Tpo media for 12–15 days. Every 3–4 days, one-third of the supernatant was replaced with fresh Tpo media and viral release was assayed by p24 ELISA (Coulter Corp.).

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REFERENCES


