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Intrinsic Human Immunodeficiency Virus Type 1 Resistance of Hematopoietic Stem Cells Despite Coreceptor Expression

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Interactions of human immunodeficiency virus type 1 (HIV-1) with hematopoietic stem cells may define restrictions on immune reconstitution following effective antiretroviral therapy and affect stem cell gene therapy strategies for AIDS. In the present study, we demonstrated mRNA and cell surface expression of HIV-1 receptors CD4 and the chemokine receptors CCR-5 and CXCR-4 in fractionated cells representing multiple stages of hematopoietic development. Chemokine receptor function was documented in subsets of cells by calcium flux in response to a cognate ligand. Productive infection by HIV-1 via these receptors was observed with the notable exception of stem cells, in which case the presence of CD4, CXCR-4, and CCR-5, as documented by single-cell analysis for expression and function, was insufficient for infection. Neither productive infection, transgene expression, nor virus entry was detectable following exposure of stem cells to either wild-type HIV-1 or lentivirus constructs pseudotyped in HIV-1 envelopes of macrophage-tropic, T-cell-tropic, or dualtropic specificity. Successful entry into stem cells of a vesicular stomatitis virus G protein-pseudotyped HIV-1 construct demonstrated that the resistance to HIV-1 was mediated at the level of virus-cell membrane fusion and entry. These data define the hematopoietic stem cell as a sanctuary cell which is resistant to HIV-1 infection by a mechanism independent of receptor and coreceptor expression that suggests a novel means of cellular protection from HIV-1.

Chemotherapeutic approaches to treatment of human immunodeficiency virus type 1 (HIV-1) infection have markedly reduced the levels of replicating virus in infected persons and have resulted in clinically demonstrated benefits in patient survival. The extent to which virus suppression leads to improved immune cell parameters is variable (1), however, and impairment of cellular regeneration may be due to alterations in organs of cell development (17, 23, 32, 33), ongoing destruction of highly infectible subsets of developing hematopoietic cells (16, 34), or defects in stem cell function (41). Efforts to overcome these abnormalities include the use of cells genetically engineered to be resistant to HIV-1 infection. The use of autologous stem cells with multilineage capability is particular appealing but is complicated by both the possibility of HIV-1 infection being already present in the cells (15) and the difficulty of transducing quiescent cells. Lentivirus vectors have been proposed as a means of overcoming the latter obstacle due to their lack of dependence on the cell cycle for integration into target cells (25, 30). These vectors have largely been constructed by using the HIV-1 backbone. Therefore, defining the issue of HIV-1 infection of stem cells may improve our understanding of stem cell dysregulation in AIDS and has practical implications for stem cell gene therapy for AIDS and the use of lentivirus vectors for the transfer of genes into stem cells.

HIV-1 entry into host cells is mediated through interaction of the virus envelope with cell surface CD4 and specific receptors of the chemokine family (4, 7, 10, 11, 14). The presence of CD4 has been reported for at least a subset of $CD34^+$ human bone marrow cells (2, 19, 24, 41) and is postulated to account for the inhibitory effects of HIV gp120 on hematopoiesis reported by some investigators (41). It has recently been reported that RNAs for the chemokine receptors CCR-5 and CXCR-4 can be detected in $CD34^+$ cells from some individuals by PCR (9), while other investigators have not detected expression of chemokine receptors or evidence of virus production in long-term culture (LTC) (38). We sought to define these issues, assessing for the presence of functional HIV-1 receptors, the impact of HIV-1 interaction with the cells, and the ability of the receptors to serve as portals of entry for virus in the stem cell subset.

MATERIALS AND METHODS

Cell separation. Bone marrow aspirates and peripheral blood were collected from normal adult donors after informed consent was obtained according to the guidelines established by the Human Investigation Committee of Massachusetts General Hospital. Low-density cells obtained by Ficoll-Hypaque (Pharmacia, Piscataway, N.J.) density centrifugation were further fractionated into different subsets ($CD34^+$, $CD34^+$ $CD38^+$, and $CD34^+$ $CD38^-$ cells from bone marrow and $CD4^+$ and $CD3^+$ $CD4^+$ cells from peripheral blood) by magnetic bead immunoselection (Miltenyi Biotec, Auburn, Calif., and Dynal, Oslo, Norway) and cell sorting (FACS Vantage; Becton Dickinson, San Jose, Calif.). G_0 or cytokine-nonresponsive stem cells were derived from $CD34^+$ cells which were cultured in Iscove's modified Dulbecco medium supplemented with 200 μg of 5-fluorouracil (Pharmacia Inc., Kalamazoo, Mich.)/ml, 100 ng of kit ligand (SCF; R&D Systems, Minneapolis, Minn.)/ml, 100 ng of interleukin-3 (R&D Systems)/ml, and 10% fetal calf serum (FCS; Sigma Chemical Co., St. Louis, Mo.) for 7 days and subsequently were sorted by flow cytometry (Becton Dickinson) based on annexin V (Caltac Laboratories, San Francisco, Calif.) and propidium iodide

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(Sigma) or 7-aminoactinomycin D (Calbiochem, La Jolla, Calif.) staining. Stem cells were also isolated based on staining with Hoechst 33342 (Molecular Probes, Eugene, Oreg.) and Rhodamine 123 (Sigma) as previously described (18), with incubation at 37°C with 10 μM Hoechst 33342 (Hst) for 40 min and with 1 μg/ml Rhodamine 123 for 20 min prior to washing and staining of cells with phycoerythrin (PE)-conjugated anti-CD34 (Becton Dickinson). Cell sorting was performed with a 488-nm light for excitation of both Rhodamine 123 and PE and a 350-nm light for Hoechst 33342.

Phenotypic analysis. Low-density cells were washed and blocked in 0.5% human immunoglobulin G (IgG; Sigma) for 10 min at 4°C prior to being washed and resuspended in phosphate-buffered saline PBS containing 0.2% bovine serum albumin (BSA) and purified mouse anti-human monoclonal antibodies (anti-CCR-5 [10 μg/ml; LeukoSite, Inc., Cambridge, Mass.] and anti-CXCR-4 [20 μg/ml; NIH AIDS Research and Reference Reagent Program, Rockville, Md.]) and incubated for 15 min at 4°C. The cells were then washed and incubated with fluorescein isothiocyanate (FITC)-conjugated affinity-purified $F(ab')_2$ goat anti-mouse IgG (heavy and light chains; Tago, Burlingame, Calif.) for 15 min at 4°C prior to being blocked with 1% normal mouse serum (Accurate Chemical & Scientific Co., Westbury, N.Y.) or mouse IgG (Caltac Laboratories) for 15 min at 4°C and stained with PE-conjugated anti-CD38 and peridinin chlorophyll protein (PerCP)-conjugated anti-CD34 antibody (Becton Dickinson) for 15 min at 4°C. After being washed, cells were suspended in PBS or fixed in 1 to 2% paraformaldehyde and analyzed by flow cytometry.

To examine receptor expression on G_0 stem cells, isolated cells were incubated with bead-conjugated mouse anti-human CD4 antibody (Dynal) for 30 min at 4°C or with unconjugated anti-CXCR-4 or -CCR-5 antibodies (Pharmingen, San Diego, Calif.) for 30 min, followed by bead-conjugated sheep anti-mouse IgG (Dynal) for 30 min at 4°C, and then scored by inverted-microscope evaluation. Bead-conjugated anti-CD19 antibody or sheep anti-mouse IgG (Dynal) was used as a negative control. Peripheral blood mononuclear cells (PBMC), macrophages, or Jurkat cells were used as a positive control. Cells were evaluated by independent readers and scored as positive or negative based on the rosetting of the immunomagnetic beads around the cells. Cells with at least three beads were considered positive.

Calcium flux. Purified $CD34^+$ cells were loaded with Indo-1/AM (Molecular Probes) and labeled with FITC-conjugated anti-CD38 and PE-conjugated anti-CD34 antibody as described elsewhere (31). NIH 3T3 cells were used as a negative control. Cells were analyzed by flow cytometry (Coulter, Hialeah, Fla.) for 30 s prior to stimulation in order to collect a baseline emission value for Indo-1/AM. Fluorescence-activated cell sorter FACS analysis was continued for up to 5 min after addition of the chemokine ligands.

Calcium flux on G_0 stem cells was measured with a Meridian Instruments (Okemos, Mich.) model ACAS570 Ultima interactive laser cytometer as described elsewhere (12, 42). Briefly, cells were washed and suspended in 10 mM HEPES buffer (pH 7.4) containing 121 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$ and 5 mM D-glucose. The cells were washed three times after 0.5 h of incubation at 37°C with 2 μM Fluo-3/AM (Molecular Probes) and suspended in fresh buffer. Laser-based microscopy was used to monitor Ca^{2+} signals in single G_0 cells at excitation and emission wavelengths of 488 and >510 nm, respectively. Digitized cell images were collected before and after stimulation with recombinant human chemokine at the concentrations noted below; the images were in pseudocolor format. Alternatively, integrated fluorescence values at an excitation wavelength of 350/380 were derived by using a SCAN1 UV scanner (Photon Technology International, South Brunswick, N.J.) and Felix software to generate a graphic display. Cells were suspended in 0.5 ml of loading buffer containing 5 mM Fura-2 (Molecular Probes) and then incubated at 37°C for 45 min. After being washed three times, the cells were suspended in 1% methycellulose loading buffer to prevent cell movement on the slide, visualized with a Zeiss model IM35 microscope, and analyzed prior to and following chemokine addition. Recombinant human macrophage inhibitory protein 1α (MIP-1α), MIP-1β, or RANTES (PeproTech, Rocky Hill, N.J.) was added at 200 ng/ml, SDF-1β (Genetic Institute, Cambridge, Mass.) was added at 1 μg/ml, and IP-10 (PeproTech) was added at 100 ng/ml.

HIV-1 infection. Cells were exposed to macrophage-tropic (M-tropic) HIV-$1_{Ba\text{-}L}$ (8.05×10^6 50% tissue culture infective doses [$TCID_{50}$/ml), T-cell-tropic (T-tropic HIV-1_{IIIB} (5.40×10^5 $TCID_{50}$/ml), or heat-inactivated (56°C for 30 min) controls. In some experiments, pHIvec2.GFP, which was created from p653RtutpC (27, 28) by substitution of humanized green fluorescent protein (GFP) for the chloramphenicol acetyltransferase gene, was used. This construct was pseudotyped in envelopes derived from HIV-1 strain HXB2 (29), YU2, or 89.6 (35) or vesicular stomatitis virus (VSV) surface glycoprotein (provided by T. Friedmann) (39). Infections were conducted at a multiplicity of infection of 1 (for strain Ba-L or IIIB) or 10 (for all of the pseudotyped viruses) in RPMI medium with 20% FCS for 24 h at 37°C in a 5% CO_2 incubator. To remove contaminating viral DNA or host cellular DNA from virus preparations, RNase-free DNase (Boehringer Mannheim Corporation, Indianapolis, Ind.) was added, with incubation for 1 h at room temperature for virus supernatants or for 45 min at 37°C for infected cells. Non-DNase-treated virus was used for functional long-term culture–initiating cell (LTC-IC) and apoptosis analyses. After being washed five times in PBS, cells were collected for DNA PCR, virus production assays, and LTC-IC and apoptosis analyses as described below.

Inhibition of HIV-1 entry. Cells were preincubated for 60 min at 37°C with recombinant human chemokines for CCR-5: MIP-1α, MIP-1β, and RANTES, each at concentrations of 500, 250, 125, 62.5, 31.25, and 0 μg/ml. Cells were then exposed to HIV-$1_{Ba\text{-}L}$ as described above and collected for DNA PCR analysis and determination of p24 production.

HIV-1 DNA PCR. Cells were lysed with a 100-μg/ml solution of proteinase K (Boehringer Mannheim Corporation) in PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], and 2.5 mM $MgCl_2$; Perkin-Elmer, Foster City, Calif.) by incubation at 60°C for 1 h and then 10 min at 95°C. Cell lysate was aliquoted for analysis of DNA by PCR with HIV-1 *gag*-specific primers SK100 and SK104 (26) for 15 cycles of 30 s at 90°C, 30 s at 55°C, and 1 min at 72°C, using a Gene Amp PCR system (model 9600; Perkin-Elmer). One-tenth of that PCR product was used for nested amplification with a second pair of *gag*-specific primers, SK38 and SK39 (26), for 20 cycles of 30 s at 95°C, 30 s at 65°C, and 1 min at 72°C. An identical protocol was used for long terminal repeat amplification with the M667-BB301 and M667-AA55 primer pairs as described elsewhere (40) or for control β-actin amplification. Semiquantitative PCR was performed by limiting dilution of Ach-2 cells (one copy of the HIV genome per cell) in uninfected PM1 cells. PCR products were subjected agarose gel electrophoresis, imaged by ethidium bromide staining, transferred to a nylon membrane, and hybridized with ^{32}P-labeled, HIV-specific probe SK19 (26) or TC1 (AAGCTTGCCTTGAGTGCTTCAAG TAGTGTGTGCCGTCTG) or with β-actin-specific probes under conditions specified below.

Poly(dT) RT-PCR. One or 50 to 200 cells (depending on the experiment) were used for reverse transcription (RT)-PCR involving oligo(dT)-primed RT followed by poly(A) tailing via a terminal-transferase-catalyzed reaction to generate a 5′ oligo(dT) transcript-poly(A)-3′ first-strand cDNA and subsequently amplified with oligo(dT) primers as previously described (3, 5, 6). The PCR product was aliquoted and used to generate Southern blots as described above. The nylon membranes were hybridized to ^{32}P-radiolabeled 3′ oligonucleotide probes for human CCR-5 (ACAGCCTGGGCTGGGGGTGGGGTGGGAGAGGTCTTT TTTA), CXCR-4 (GGAGTGGGTTGATTTCAGCACCTACAGTGTACAGT CTTGT), CD4 (CCACGCCATTTCCTTTTCCTTCAAGCCTAGCCCTTCTC TC), or glyceraldehyde-3-phosphate dehydrogenase (Gibco BRL, Gaithersburg, Md.) in Express hybridization solution (Clontech, Palo Alto, Calif.) at 37°C with a final wash performed in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–sodium dodecyl sulfate 0.1% at 37°C. Phosphorimager (Bio-Rad, Hercules, Calif.) analysis was used to quantitate the signal intensity.

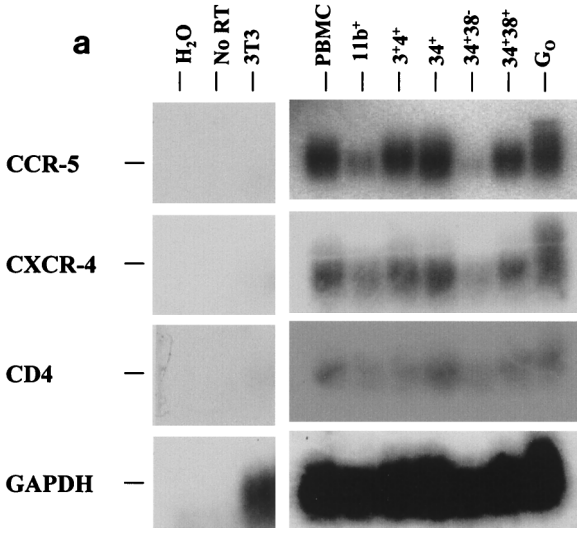
Measurement of HIV-1 production. After 24 h of exposure to HIV-1 Ba-L, cells were washed a minimum four times with PBS and cocultured with PM1 or H9 cells in RPMI 1640 with 20% FCS. Medium was collected on days 4, 7, 10, 14, 21, 28, and 35 for analysis with an HIV-1 p24 enzyme-linked immunosorbent assay kit (Coulter Corporation), performed according to the manufacturer's protocol.

LTC-IC. The presence of LTC-IC was assessed by limiting dilution of the cells on an autologous bone marrow stromal feeder layer for 5 weeks as previously described (36). Briefly, low-density bone marrow cells were cultured at 37°C for 3 to 4 days prior to transfer to 33°C in LTC medium (alpha minimal essential medium with 12.5% horse serum, 12.5% FBS, 0.2 mM *i*-inositol, 20 mM folic acid, 10^{-4} M 2-mercaptoethanol, 2 mM L-glutamine [StemCell Technologies Inc., Vancouver, British Columbia, Canada], and 10^{-6} M hydrocortisone [Sigma]). A confluent stromal feeder layer was trypsinized, irradiated (15 Gy), and subcultured in 96-well flat-bottomed plates at a density of 1.25×10^4 cells/well. Within 1 week, $CD34^+$ cells or HIV-1-exposed $CD34^+$ cells were seeded at a density of 1,000, 500, 250, 125, 63, 31, or 0 per well with 24 replicate wells per cell concentration and then cultured at 33°C for 5 weeks with half-volume medium changes weekly. The culture plates were then centrifuged. Iscove's modified semisolid Dulbecco medium (StemCell Technologies) containing 0.9% methyl cellulose, 30% FBS, 1% BSA, 10^{-4} M 2-mercaptoethanol, and 2 mM L-glutamine and supplemented with 20 ng of interleukin-3, 20 ng of granulocyte-macrophage colony-stimulating factor per ml, 50 ng of kit ligand (R&D systems), 20 ng of granulocyte colony-stimulating factor, and 3 U of erythropoietin (Amgen Inc., Thousand Oaks, Calif.)/ml was overlaid. Following 2 to 3 weeks of incubation at 37°C in a 5% CO_2 atmosphere, colonies were quantitated by phase-contrast microscopy and the LTC-IC frequency was calculated.

Detection of apoptosis. Control or HIV-1-exposed $CD34^+$ cells were washed, suspended in PBS containing 0.2% BSA, and incubated with FITC-, PE-, or PerCP-conjugated antibody for 15 min at 4°C. After being washed, the cells were suspended in 100 μl of Ca^{2+}-enriched binding buffer (R&D Systems) containing 100 μl of a 5-μg/ml solution of 7-aminoactinomycin D (Calbiochem) and 5 μl of a 200-μg/ml solution of annexin V-FITC (Caltac) and then incubated for 15 min at room temperature prior to addition of 500 μl of Ca^{2+}-enriched binding buffer and analysis by flow cytometry (Becton Dickinson). Cells staining with annexin V were considered apoptotic (13).

RESULTS

Receptor and coreceptor mRNA expression detected in multiple hematopoietic cell subsets. Cells representing particular stages of blood cell development in the adult human were isolated by flow-cytometric or functionally based systems that



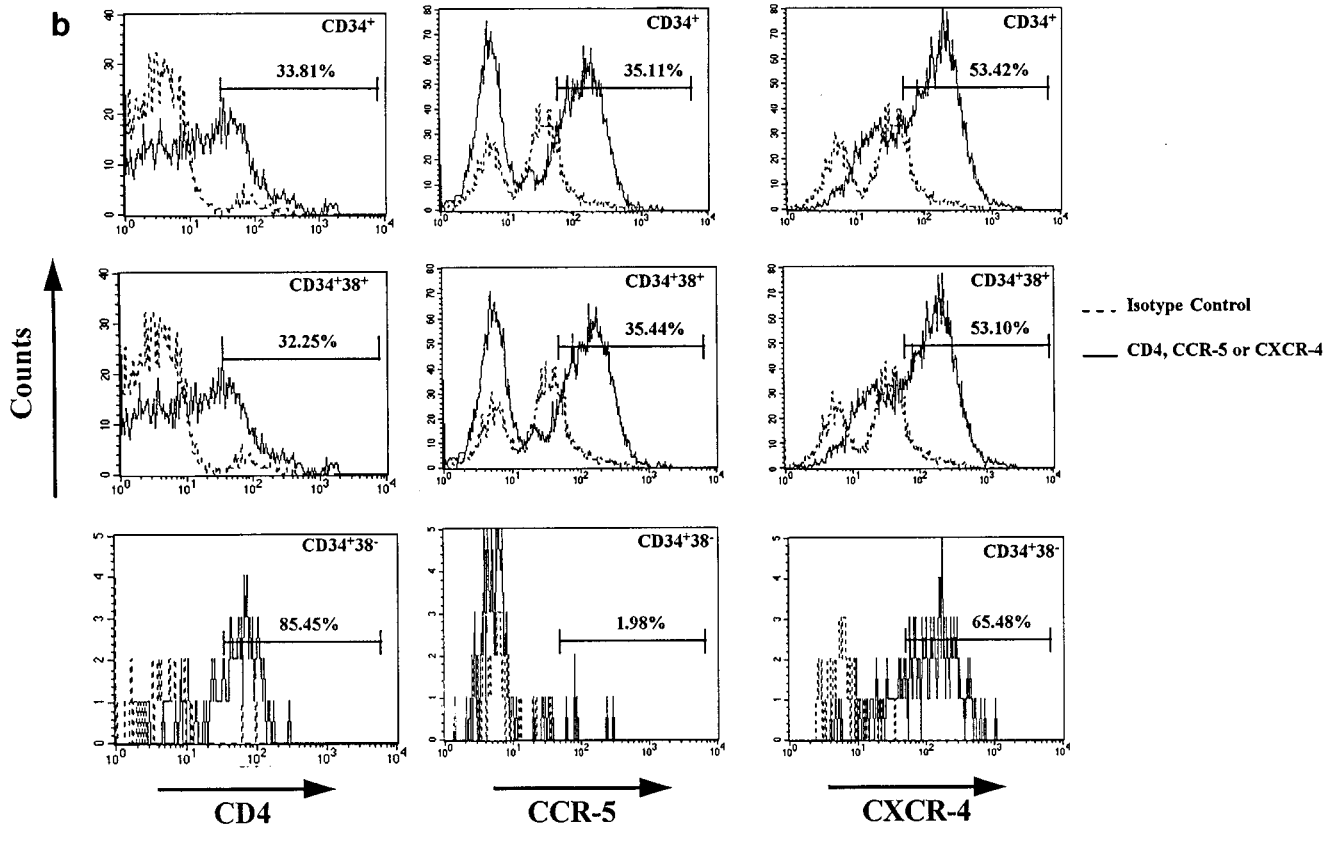


FIG. 1. HIV-1 receptors and coreceptors are expressed in human hematopoietic progenitors. (a) Detection of CCR-5, CXCR-4, and CD4 mRNAs in bulk $CD34^+$ subsets (50 to 200 cells/well) by the poly(dT) RT-PCR technique described in Materials and Methods. cDNA from the RT-PCR yielded products of various lengths which were aliquoted, electrophoresed, and probed with the radiolabeled probes indicated. The probed membranes were washed and imaged by phosphorimager analysis. (b) Determination of the presence of CD4, CCR-5, and CXCR-4 proteins on the cell surface of $CD34^+$ subsets by flow cytometry. Staining with specific monoclonal antibodies reactive against the indicated antigens (solid lines) was compared with that of isotype controls (dashed lines) on either bulk $CD34^+$ or the subsets indicated. Gates were set to exclude ~90% of isotype control stained cells and percent positivity calculated beyond that indicated marker. The irregular contours in the $CD34^+$ $CD38^-$ subset analysis are due to small cell numbers. (c) Calcium flux in $CD34^+$ subset cells after stimulation with chemokines as assessed by FACS analysis of Indo-1/AM-loaded cells. Baseline measurements were performed prior to addition of the indicated chemokine (arrow), and the fractions of cells staining with the indicated antigen-specific antibodies and Indo-1/AM were calculated over time as indicated.

have been previously described (3, 8, 18, 37). The stem cell population was isolated by the previously described method of selectively killing more-mature cells, thereby enriching for a cytokine-nonresponsive subset with stem cell-like characteristics. Cells were assessed for expression of CD4 and chemokine receptors CCR-5 and CXCR-4 mRNA and protein by techniques adapted for the small numbers of primary cells available from standard donations. For mRNA expression, a poly(T)-primed RT-PCR technique which has been shown to permit detection of multiple polyadenylated transcripts from a single reaction tube was used, thereby allowing internal normalization (6). Low levels of message for CD4 were detectable by RT-PCR in PBMC, myelomonocytic cells ($CD11b^+$), mature T cells ($CD3^+$ $CD4^+$), heterogeneous hematopoietic progenitor cells ($CD34^+$), lineage-committed hematopoietic progenitor cells ($CD34^+$ $CD38^+$), primitive hematopoietic progenitor cells ($CD34^+$ $CD38^-$), and stem cells (G_0) but not in NIH 3T3 controls (Fig. 1a). Similarly, message for CXCR-4 and CCR-5 was detectable in each hematopoietic cell type tested, although CCR-5 levels in $CD34^+$ $CD38^-$ cells appeared to be lower, as confirmed with multiple independent samples ($n = 4$). To more precisely define the presence of the receptor transcripts

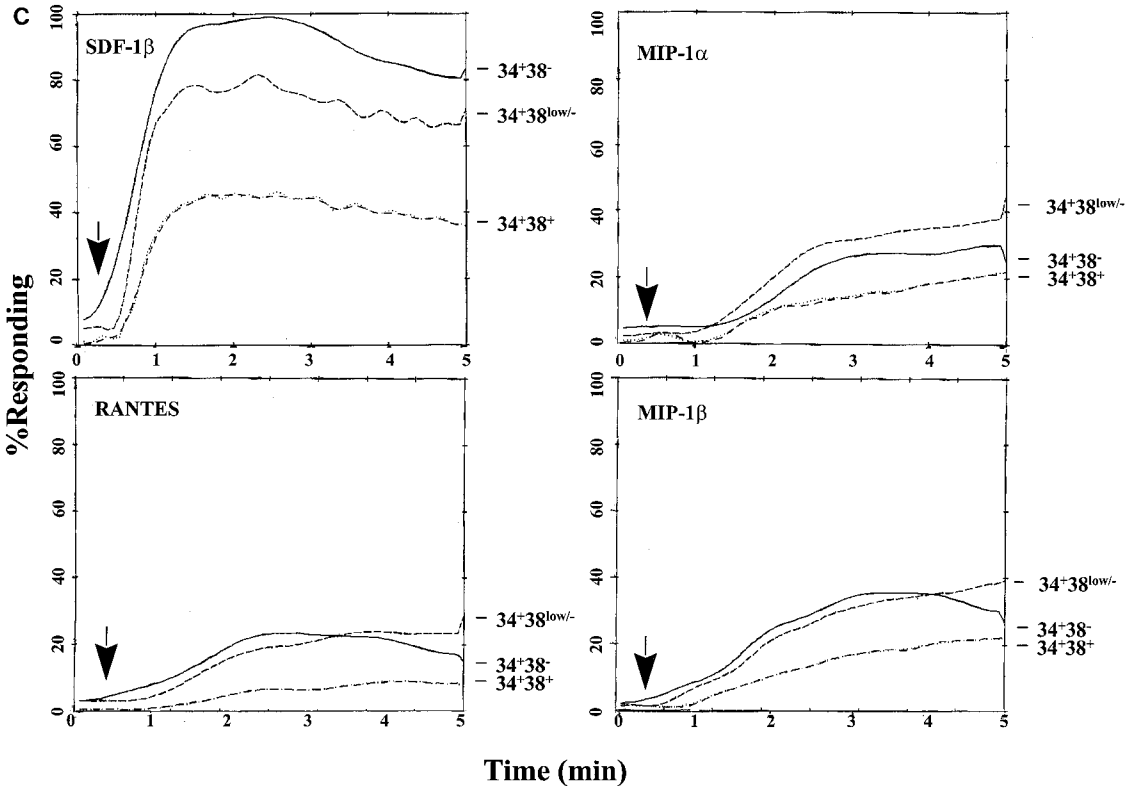


Fig. 1—*Continued.*

in stem cells, individual cells were isolated by micromanipulation and single-cell RT-PCR profiles were generated as previously described (5, 6). The cells consistently demonstrated detectable CD4, CXCR-4, and CCR-5 message compared with controls; the results for five representative cells are shown in Fig. 2a.

Functional coreceptor expression is differentiation stage specific. The presence of protein produced from the receptor transcripts was assessed by specific antibody staining and, independently for chemokine receptors, by calcium flux analysis. Anti-CD4 staining, analyzed by flow cytometry, indicated that CD4 expression occurred in subpopulations of $CD34^+$ cells (Fig. 1b), similar to the findings reported by others (2, 19, 24, 41). CCR-5- and CXCR-4-specific antibodies stained fractions of relevant $CD34^+$ cells (Fig. 1b), with only minimal staining of $CD34^+$ $CD38^-$ cells by the anti-CCR-5 antibody, consistent with the low transcript levels observed.

Chemokine signaling, as measured by the generation of a calcium flux in cells bearing cognate receptors, was used as a functional assessment of chemokine receptors. Responsiveness of various subsets of $CD34^+$ cells to SDF-1 (the ligand for the CXCR-4 receptor) and to RANTES, MIP-1α, and MIP-1β (ligands for the CCR-5 receptor) was measured on Indo-1/AM-loaded cells by FACS analysis (Fig. 1c). NIH 3T3 cells were used as a cell control, IP-10 (the ligand for CXCR-3) was used as a chemokine control, and measurements were taken over time, using the cells prior to and following exposure to chemokine to establish a target cell baseline control. Response to SDF-1 was substantial in all populations of $CD34^+$ cells, although increased response was noted in the $CD34^+$ $CD38^-$ cells despite the lack of a difference in the frequency of CXCR-4 surface protein in that subfraction compared with $CD34^+$ $CD38^+$ cells. Similarly, the relationship between detectable surface protein for CCR-5 and the response to ligand was not direct. Despite low levels of message and surface CCR-5 in the $CD34^+$ $CD38^-$ subset, there was a calcium flux approximately equivalent to that of other cell fractions when cognate ligands were applied. NIH 3T3 cells did not demonstrate calcium flux, and IP-10 did not induce calcium flux except in a human CXCR-3-expressing cell line.

Determination of stem cell coreceptor production and function. The rarity of stem cells (G_0) precluded the routine use of flow cytometry, and thus we developed an immunomagnetic bead rosette assay (Fig. 2b). This assay utilizes the binding of specific monoclonal antibodies to target epitopes on cells, as in immunofluorescence assays. However, instead of fluorescein conjugation, immunomagnetic bead conjugation was used as a means of enhancing the ability to detect antibody binding by microscopy; the size of the Dynal beads permitted ready enumeration of rosetted cells, and there was a low frequency of nonspecific binding (0.7 to 6%) when the second step alone or irrelevant-antibody-conjugated beads were used. The estimated frequencies of CD4-, CXCR-4-, and CCR-5-expressing cells compared with bead-alone or irrelevant-antibody-conjugated bead controls (specific minus nonspecific binding) were 12.2, 23.2, and 23.6%, respectively (Table 1). The use of a large-scale stem cell preparation, generated by pooling multiple independent marrow preparations, permitted flow-cytometric analysis of $CD34^+$ $CD4^+$ cells and demonstrated a high level of coexpression of CXCR-4 and CCR-5 compared with the isotype control (Fig. 2b).

Single-cell digital fluorescence imaging was used to document stem cell (G_0) calcium flux in response to chemokines. MIP-1α, MIP-1β, and SDF-1 generated evidence of calcium flux (Fig. 2c) and thereby confirmed the functional status of surface CCR-5 and CXCR-4 on stem cells. In contrast, IP-10

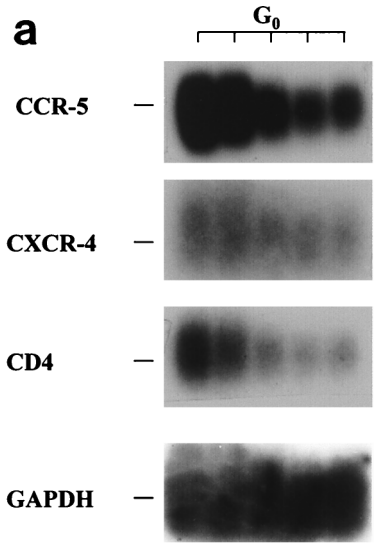
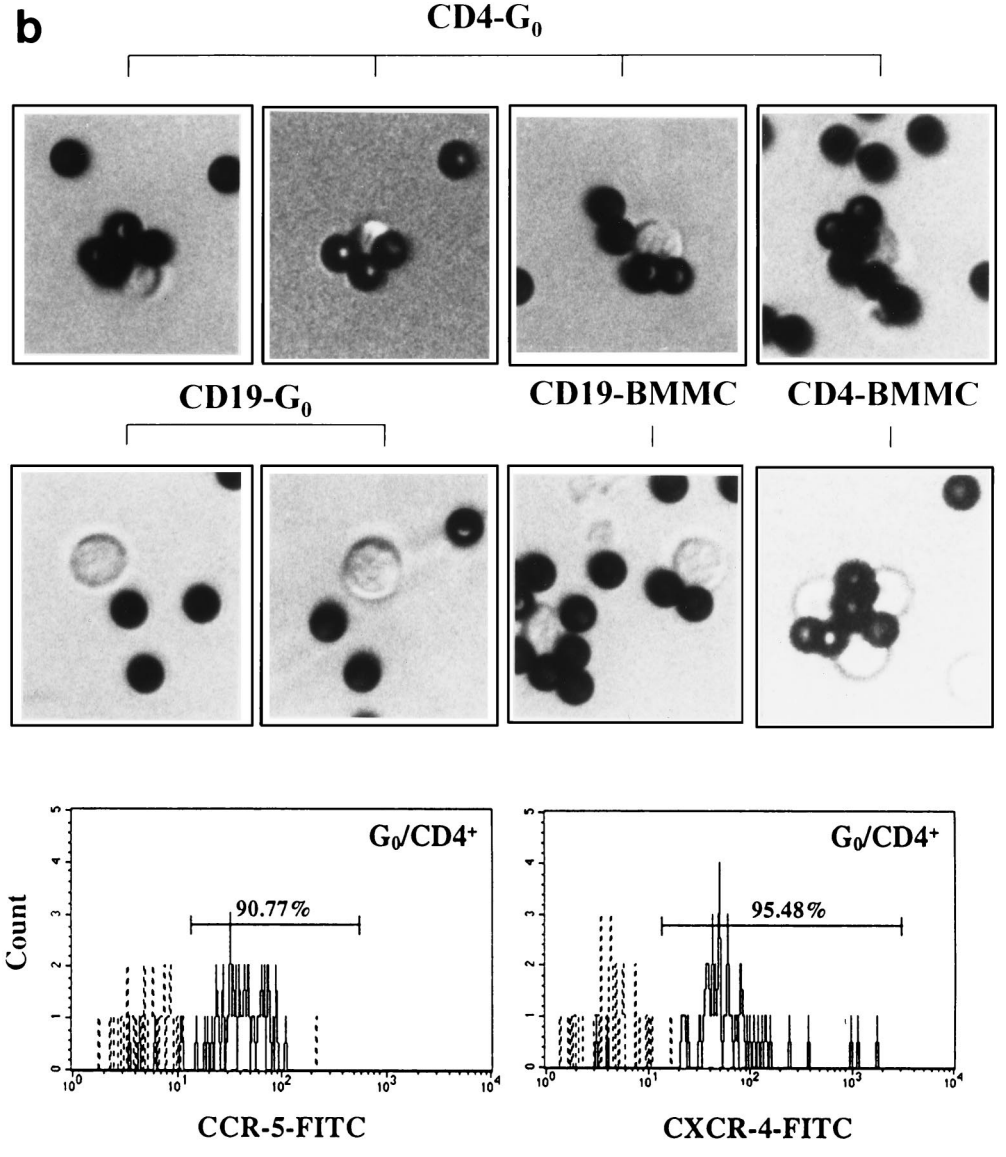
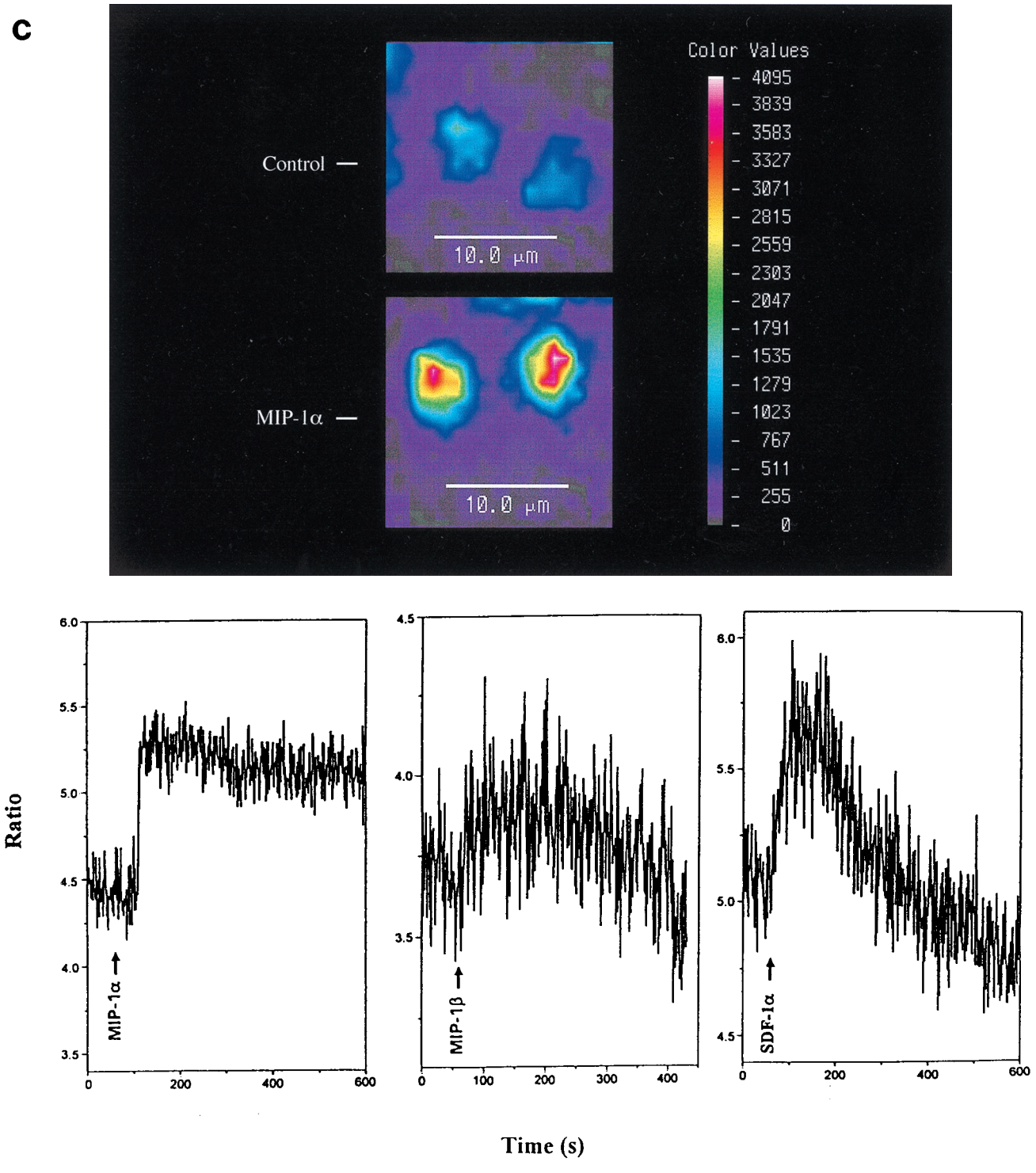


FIG. 2. Stem cells coexpress CD4 and functional chemokine receptors. (a) Individual G_0 cells were isolated by micromanipulation and assayed for gene expression by single-cell RT-PCR as described in the text. The RT-PCR product from an individual cell (each of five, indicated by the tick marks) was aliquoted, electrophoresed, and probed with radiolabeled probes for the indicated genes. The results indicate that CD4, CCR-5, and CXCR-4 mRNAs from the same individual stem cell can be detected. (b) Analysis of protein expression on stem cells was performed by using an immunomagnetic bead rosetting assay as described in the text. Immunomagnetic beads (dark circles) were assessed for their ability to bind to target cells by two independent readers, using phase-contrast microscopy (original magnification, ×40). The upper panels demonstrate the binding of representative CD4-specific beads to G_0 stem cells. The middle panels demonstrate the lack of CD19-bead binding to G_0 stem cells (negative control) or beads binding to peripheral blood lymphocytes (positive controls). For the lower panels, G_0 cells selected for CD4 were pooled and flow-cytometrically analyzed for staining with either CXCR-4 or CCR-5 or isotype controls. (c) Calcium flux in G_0 stem cells after stimulation with MIP-1α, MIP-1β, or SDF-1 as assessed by laser-based digital fluorescence microscopy. The upper image represents individual cells responding to stimulation (as indicated by color coding for fluorescence intensity), while quantitative graphic depictions of the fluorescence increase over time in fields of ~10 cells each are shown in the lower panels.





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did not induce flux in stem cells but did induce calcium flux in human CXCR-3-transduced control cells.

Chemokine receptors function as HIV-1 coreceptors on CD34$^+$ cell subsets except G_0 stem cells. Definition of the functional characteristics of the coreceptor molecules was further pursued through exposure of cells to stocks of infectious HIV-1. Given the presence of identifiable receptors for M-tropic strains (utilizing CCR-5) and T-tropic strains (utilizing CXCR-4), appropriate virus envelopes were used (HIV-$1_{Ba\text{-}L}$ and HIV-$1_{HxB\text{-}2}$, respectively). Following exposure to concentrated stocks of virus, infected cells were evaluated for (i) the presence of HIV DNA, indicating virus entry and reverse transcription; and (ii) the production of HIV-1 p24 antigen following addition of highly infectible cell lines, indicating comple-

TABLE 1. HIV-1 receptor/coreceptor expression by immunomagnetic bead rosette assay

Cell line	% of cells expressing:		
	CD4	CCR-5	CXCR4
G_0	12.2	23.2	23.6
Jurkat	13.1	5.4	87.5
Macrophage	ND[a]	56.6	ND

[a] ND, not determined.

tion of a replicative virus life cycle and passage of virus. Virus DNA was detectable at the level of a single cell diluted 10^{-4} in titration experiments of Ach-2 cells containing a single proviral copy per cell (data not shown). HIV-1 DNA was evident in all subsets of cells exposed to infectious, but not heat-inactivated, virus, with the notable exception of the G_0 stem cells (Fig. 3a). G_0 cells independently isolated from independent normal donors (three are shown) were consistently negative for viral DNA. $CD34^+$ cell fractions other than stem cells had detectable viral DNA which was inhibitable at a level of approximately 50% by preincubation with cognate ligands for CCR-5, MIP-1α, MIP-1β, and RANTES (data not shown). The nonstem cell fractions also demonstrated productive completion of the virus life cycle by passage of HIV-1 to the readily infectible indicator cell lines PM-1 (Fig. 3b) and H9 (data not shown) when HIV-$1_{Ba\text{-}L}$ or HIV-$1_{HxB\text{-}2}$, respectively, was used. Prompt production and passage of virus to PM-1 from the relatively mature $CD34^+$ $CD38^+$ population of cells was noted. While $CD34^+$ $CD38^-$ cells had clearly identifiable virus DNA present, passage of infectious virions was very delayed, with p24 antigen being detectable upon cocultivation with PM-1 only after prolonged periods (~21 days). In no instance was p24 detectable upon cocultivation of indicator (PM-1 or H9) cells with virus-exposed G_0 cells, including experiments extended out to 35 days.

In vivo corroboration that stem cells are resistant to HIV-1 infection. To determine if stem cells were infected in vivo, bone marrow samples were obtained from HIV-1-infected patients with high levels of circulating virus and low blood cell counts. Using multiple independent patient samples, HIV DNA was identified in PBMC and bone marrow mononuclear cells (BMMC), but there was consistently no detectable HIV DNA in G_0 cells (Fig. 4a) ($n = 7$).

Stem cell function is not altered following exposure to HIV-1. Functional characteristics of stem cells could theoretically be perturbed despite the absence of productive infection and, if apoptosis were induced, could affect the ability to detect infection. To address these issues, infectious HIV-1 was added to LTC systems used to quantitate cells with stem cell characteristics (LTC-IC). HIV-1 production was noted in the system (Fig. 4b) due to the heterogeneous population of input cells, but no impact on the quantitative yield of stem cell function was detected (Fig. 4c). Further, analysis for apoptosis by the annexin V assay did not demonstrate any induction of apoptosis among $CD34^+$ subsets (data not shown).

Stem cell resistance is mediated at the level of virus fusion and entry. RT in quiescent cells may be incomplete and, in lymphocytes, may result in partial cDNA intermediates (40). To evaluate this possibility in stem cells, PCR primer pairs corresponding to the 5′ and of the primer binding site and U3 to U5 portions of the HIV-1 genome that were reverse transcribed even in quiescent lymphocyte populations were used (labeled TC-1 in Fig. 5). To assess whether the blockade to infection was at the receptor level or followed receptor interaction, and GFP-encoding HIV-1 construct pseudotyped with either T-tropic (HXB2), M-tropic (YU-2), or dualtropic (89.6) HIV-1 envelopes was compared with the same virus construct pseudotyped with the VSV G protein. VSV G protein permits virus fusion with the cell membrane via mechanisms which bypass those mediated by CD4 and CCR-5 (20). Only the VSV G-pseudotyped virus was capable of infecting the stem cell population (Fig. 5a). No HIV DNA was detectable in stem cells when HIV-1 envelopes or heat-inactivated VSV G envelope pseudotypes were used as assayed either by PCR or, for GFP expression, by fluorescence microscopy. HIV-1 envelope pseudotypes were capable of infecting Jurkat or primary mononuclear cell controls. These data demonstrate that stem cells have a block to HIV-1 infection and that the level of the blockade is in the steps of viral-cell membrane fusion and entry. Steps downstream of these event are intact, as evidenced by the VSV G-pseudotyped infection.

Stem cell resistance is independent of the method of isolation. An independent stem cell purification process using Rhodamine 123 and Hoechst 33342 staining as defined by others (18) was employed to exclude the possibility that the selection method induced alterations in the ability of the stem cells to be infected. The exclusion of Rhodamine 123 and low-intensity staining with Hoechst 33342 in $CD34^+$ cells have been shown to correlate with a population capable of functioning as stem cells in in vitro and in vivo experiments (22). Following exposure to virus, the cells corresponding to a more mature population (i.e., staining brightly with both stains) acquired detectable HIV DNA, but stem cells (staining dimly with both stains)

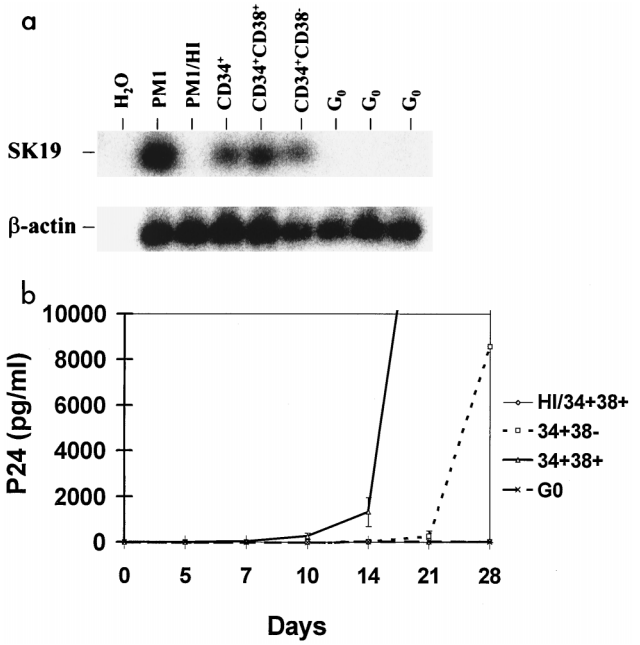

FIG. 3. HIV-1 entry and production are subset specific in hematopoietic cells. (a) M-tropic HIV-$1_{Ba\text{-}L}$ infection in vitro detected by DNA PCR in the indicated cell types. Pools of ~200 cells were assessed for HIV-1 DNA as described in the text. Three independent preparations from independent donors are shown for the G_0 subset of cells. Consistent results were obtained from independent replicates-quadruplicates of all lanes shown. (b) HIV-1 virus production in $CD34^+$ subsets and G_0 stem cells which were exposed to HIV-$1_{Ba\text{-}L}$ for 24 h, extensively washed, and subsequently cocultured with PM1 cells as an indicator cell line. HIV-1 p24 enzyme-linked immunosorbent assays were performed on culture supernatants at the indicated times. Similar results were obtained when identical experiments were performed with HIV-1_{IIIB} and with H9 cells as the indicator cell line.

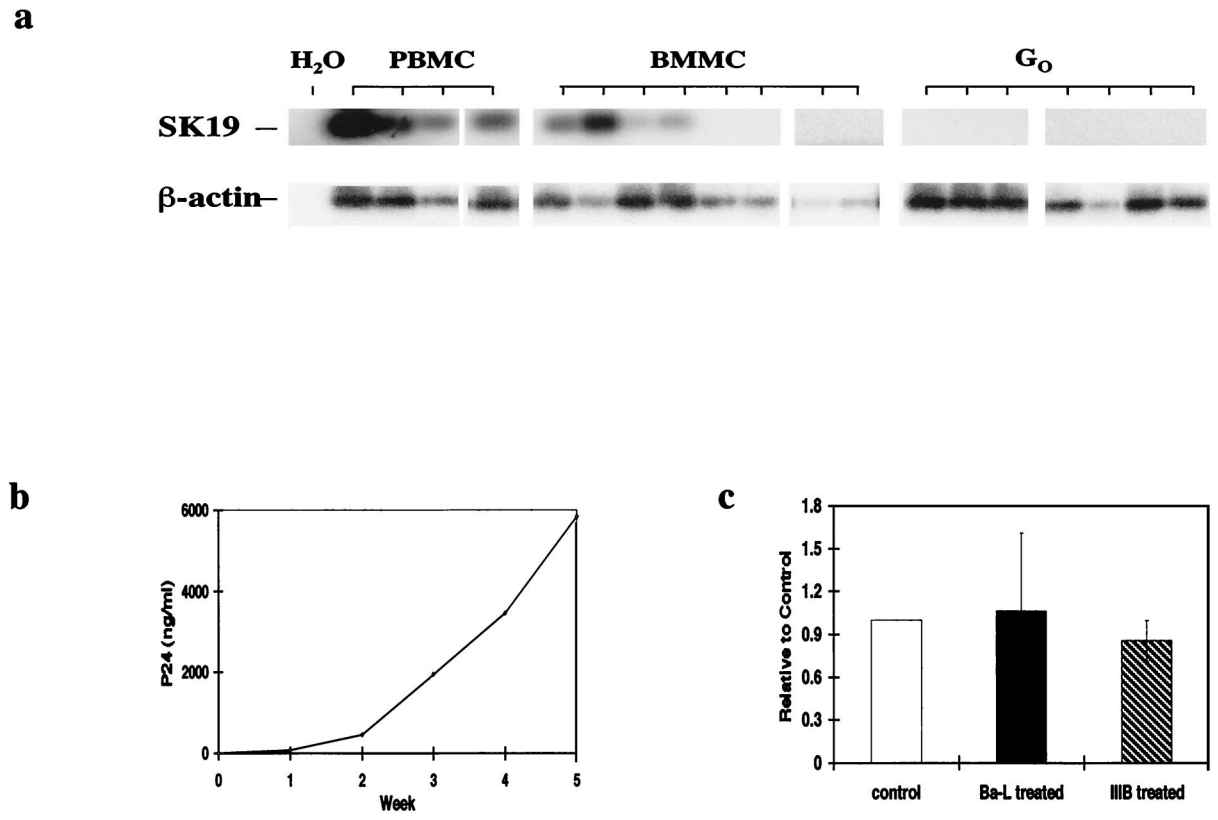

FIG. 4. Detection of the HIV-1 genome in subsets of hematopoietic cells from AIDS patients, and effect of HIV-1 on stem cell function in vitro. (a) PCR analysis of HIV-1 DNA in subsets of cells derived from individuals with advanced HIV disease and cytopenia. Each lane represents cells from an individual patient. All G_0 lanes correspond to samples in the BMMC lanes except for one patient, in which case an inadequate number of BMMC cells were obtained to proceed with G_0 isolation. PBMC samples were obtained from independent patients with similar clinical profiles. (b) Measurement of HIV-1 p24 on Ba-L-exposed $CD34^+$ cells cultivated on a bone marrow stromal cell monolayer. Similar results were seen with the use of IIIB virus. (c) Effect of HIV-1 on LTC-IC. Limiting-dilution analysis was performed on cells in the presence or absence of HIV-1 as indicated. The number of LTC-IC was derived as described in Materials and Methods, and a ratio relative to the uninfected controls was calculated. Bars represent the means ± standard errors of the means for data from three independent experiments.

had neither late nor early RT products (Fig. 5b), as was seen with G_0 cells. Further, these cells were readily infectable with virus when the envelope was of the VSV G type. These data confirm the resistance of stem cells to HIV-1 infection and demonstrate that the block can be overcome if an alternative, CXCR-4- and CCR-5-independent mechanism of envelope-cell membrane fusion is used.

DISCUSSION

There has been a longstanding controversy regarding stem cell susceptibility to HIV-1 infection that has been fueled recently by reports of chemokine receptor expression on primitive hematopoietic cells (9) and functional data failing to note detectable effects of virus on long-term stem cell culture or the ability to detect virus in those cultures (38). We sought to address this controversy on a molecular and functional level, specifically addressing which subpopulations of blood progenitor cells had the necessary surface molecules to permit virus entry, whether those molecules were functional in yielding physiologic responses to recombinant cognate ligands, and whether the ligand response and virus coreceptor functions were synonymous. It should be noted that the populations which we assessed included subpopulations distinct from those often referred to as stem cells in the gene therapy literature. In that context, $CD34^+$ cell populations are often called stem cells. While the $CD34^+$ population contains stem cells, the vast majority of that population are more-mature progenitor cells. We subdivided the $CD34^+$ population by immunophenotypic (CD38) and functional (G_0 and Rhodamine 123-Hoechst 33342 staining) characteristics in an effort to more clearly define the events occurring in the subfraction most likely to provide long-term hematopoiesis in vivo.

These studies demonstrated that all subsets of the $CD34^+$ population express CXCR-4 in at high frequencies. In contrast, CCR-5 is differentially expressed in primitive cells, with minimally detectable levels in the $CD34^+$ $CD38^-$ subset but more-readily detectable message and protein in either the more mature $CD34^+$ $CD38^+$ cells or stem cells. Further, we have found that CCR-5 is expressed exclusively on $CD34^+$ cells derived from bone marrow and not from other stem cell sources (33a). Similarly, there was a direct correlation between CXCR-4 expression and response to ligand that does not appear to be the case for CCR-5, for which variability in the relationship was more pronounced. In each case, coexpression of CD4 with the chemokine receptors was noted in at least a subset of the cells, and for all but stem cells, the receptor-coreceptor complexes appeared sufficient for virus infection. Both short-term analyses of virus entry by PCR and GFP expression and longer-term analysis of virus replication yielded positive results for cell infection except in the stem cell subset. In the case of stem cells, the presence of the CD4 receptor in conjunction with functional coreceptors was documented at

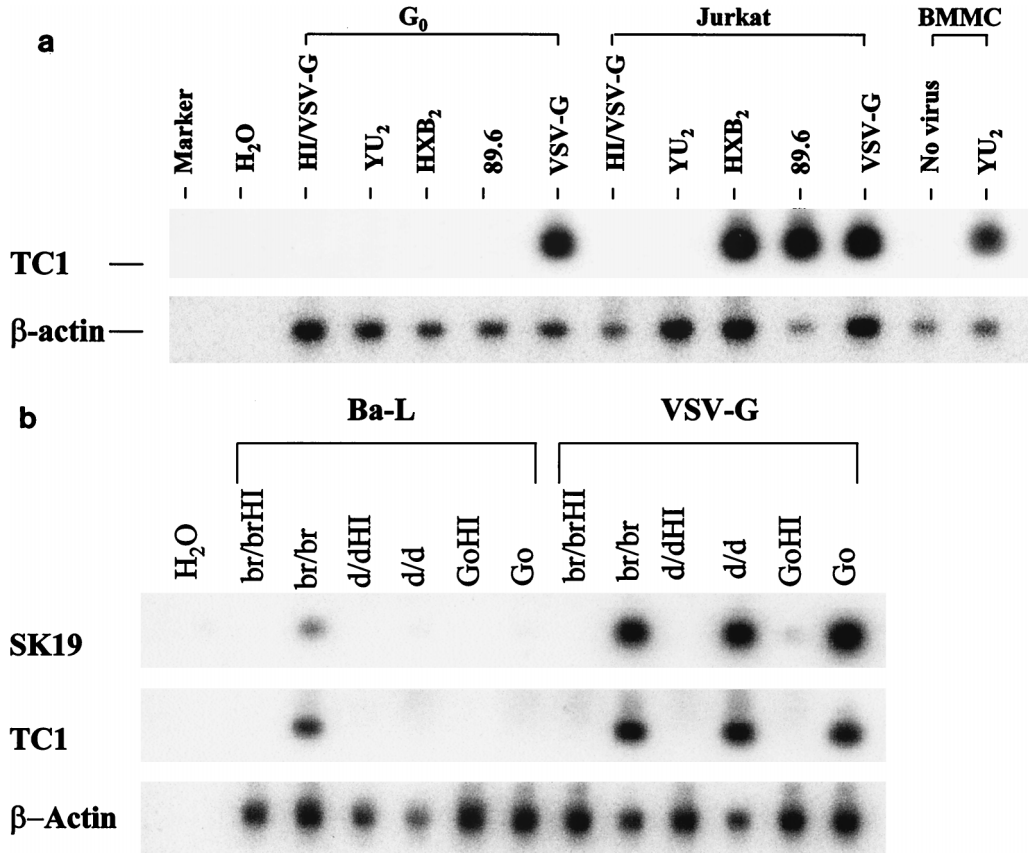

FIG. 5. Stem cell blockade to HIV-1 infection, regardless of HIV-1 tropism, is confirmed in stem cells derived by independent methods and can be circumvented by pseudotyping in VSV G-enveloped virus. (a) G_0 cells were exposed to recombinant HIV-1 pseudotyped in envelope of M-tropic (YU_2), T-tropic (HXB_2), or dualtropic (89.6) specificity or in envelope containing VSV G. Cells were analyzed by DNA PCR for early HIV transcripts. Heat-inactivated VSV G-pseudotyped virus (HI/VSV-G) was used to control for virus infectivity. Jurkat and BMMC cells were used as positive-control cell lines to control for HIV-1 enveloped virus infectivity. Lambda DNA (marker) or water alone (H_2O) was used as a PCR control. (b) Cells isolated by either Rhodamine-Hoechst staining, with bright/bright (br/br) representing more-differentiated cells and dim/dim (d/d) representing stem cells, or by cytokine nonresponsiveness (G_0) were exposed to wild-type, Ba-L, or pseudotyped HIV within a VSV G envelope and evaluated for infection by PCR directed against late (SK19) or early (TC1) RT products. HI, heat-inactivated control virus preparation. All products were confirmed by radiolabeled specific oligonucleotide hybridization as shown following phosphorimager analysis.

the single-cell level, and yet no evidence of virus entry was detected when multiple assay techniques were used.

The data demonstrate that the hematopoietic stem cell is incapable of HIV-1 infection. These results were largely derived from in vitro analyses, but the clinical relevance of the conclusion is further supported by in vivo data generated by examining stem cells isolated from patients with AIDS. The stem cell is therefore not a potential long-lived reservoir of virus and is an appropriate cell to consider for use in autologous gene therapy approaches to AIDS. To the extent that this cell can be recovered from AIDS patients, it may be envisioned to be a virus-free cell type that may be transduced with anti-HIV constructs for possible immune reconstitution. The ability to transduce the stem cell with HIV-based constructs was also documented in this study but was found to be restricted to constructs pseudotyped in VSV G envelopes. There appears to be no blockade to such constructs entering quiescent stem cells and, at least transiently, expressing a transgene. Integration of the transgene and durable expression were not tested in this study, however.

Several potential mechanisms may contribute to stem cell resistance to HIV-1 infection, such as autocrine production of a cognate chemokine, altered processing of receptors, a requirement for a third, as-yet-unidentified member of a receptor complex, or the presence of an inhibitory receptor complex constituent. Since an absolute blockade to infection was not seen with high concentrations of exogenous chemokine, it is unlikely that endogenous chemokine expression is sufficient to induce an autocrine blockade. The intact receptor signaling in response to ligand that we observed would indirectly argue against altered receptor processing, and the levels of receptor expression were comparable to those seen with infectible subsets of cells, suggesting that receptor number was not limiting (though receptor density changes cannot be excluded). Rather, we hypothesize that the basis for resistance resides in either the need for an additional receptor complex component or an inhibitory alteration of the receptor complex that is present in stem cells. The precise mechanism for the blockade is unknown, but recent reports of a subset of a hematopoietic cell line that is also resistant to HIV-1 infection while expressing receptor and coreceptor molecules (21, 22) may provide the reagents necessary to define it. While expression of CD4 and chemokine coreceptors may be necessary for HIV-1 infection, the data presented here demonstrate that it is not sufficient for all cell types. The hematopoietic stem cell is an example of a primary cell type uniquely protected from HIV-1 infection by an endogenous mechanism. Whether this mechanism can be used to protect other cell types remains to be determined.

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H.S. and T.C. contributed equally to this work.

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