A Preponderance of CCR5⁺ CXCR4⁺ Mononuclear Cells Enhances Gastrointestinal Mucosal Susceptibility to Human Immunodeficiency Virus Type 1 Infection

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The gastrointestinal mucosa harbors the majority of the body's CD4⁺ cells and appears to be uniquely susceptible to human immunodeficiency virus type 1 (HIV-1) infection. We undertook this study to examine the role of differences in chemokine receptor expression on infection of mucosal mononuclear cells (MMC)s and peripheral blood mononuclear cells (PBMCs) by R5- and X4-tropic HIV-1. We performed in vitro infections of MMCs and PBMCs with R5- and X4-tropic HIV-1, engineered to express murine CD24 on the infected cell's surface, allowing for quantification of HIV-infected cells and their phenotypic characterization. A greater percentage of MMCs than PBMCs are infected by both R5- and X4-tropic HIV-1. Significant differences exist in terms of chemokine receptor expression in the blood and gastrointestinal mucosa; mucosal cells are predominantly CCR5⁺ CXCR4⁺, while these cells make up less than 20% of the peripheral blood cells. It is this cell population that is most susceptible to infection with both R5- and X4-tropic HIV-1 in both compartments. Regardless of whether viral isolates were derived from the blood or mucosa of HIV-1-infected patients, HIV-1 p24 production was greater in MMCs than in PBMCs. Further, the chemokine receptor tropism of these patient-derived viral isolates did not differ between compartments. We conclude that, based on these findings, the gastrointestinal mucosa represents a favored target for HIV-1, in part due to its large population of CXCR4⁺ CCR5⁺ target cells and not to differences in the virus that it contains.

The intestinal mucosa contains most of the body's lymphocyte population (6, 27) and therefore likely represents the largest reservoir of human immunodeficiency virus type 1 (HIV-1) and site of viral replication. Other factors may contribute to enhance the mucosa's susceptibility to HIV-1. Since much of its immense surface area contacts a bacterium- and antigen-rich environment, the gastrointestinal mucosa is maintained in a state of "physiologic inflammation," characterized by an intrinsically high level of chemokines and other proinflammatory mediators. Peripheral blood mononuclear cells (PBMCs), including those infected with HIV-1, are therefore recruited to the intestinal mucosa (14). Further, once the virus gains access to the mucosal environment, HIV-1 replication appears to be enhanced, and CD4⁺ cells depleted. This was shown by Veazey et al., who demonstrated that within weeks of simian immunodeficiency virus (SIV) infection, administered intravenously or intrarectally, SIV rapidly depletes mucosal lymphocytes, and the mucosal lymphoid tissue contains more SIV-infected lymphocytes than the peripheral blood or other secondary lymphoid tissues (46). Selective and early depletion of mucosal CD4⁺ cells also appears to occur in HIV-1-infected humans (10, 24, 39).

Increased mucosal susceptibility to HIV-1 is thought to reflect phenotypic differences in the cellular targets for HIV-1 between compartments. Mucosal CD4⁺ T lymphocytes are

predominantly of the activated, memory phenotype, compared to the resting, naive lymphocytes that represent the vast majority of cells in blood and other nonmucosal lymphoid tissue (38, 49). Further, significantly more mucosal mononuclear cells (MMCs) express CCR5 (CD195), the chemokine receptor utilized for cellular entry by most primary HIV-1 isolates, than do peripheral blood cells (3, 23). The other major chemokine receptor utilized by HIV-1, CXCR4 (CD184), is expressed equally on MMCs and PBMCs (3, 23). Since HIV-1 predominantly uses these chemokine receptors for entry into a CD4⁺ cell and because HIV-1 replicates most efficiently in activated cells (36, 40, 42, 50), we hypothesize the virus should more readily infect and replicate in MMCs than in PBMCs. In support of this assertion, we and others have shown that more HIV p24 protein is produced from isolated MMCs than from isolated PBMCs after in vitro infection with either CCR5 (R5)-tropic or CXCR4 (X4)-tropic HIV-1 (3, 23).

Due to the error-prone nature of HIV-1's reverse transcriptase, mutations develop with each replicative cycle and over time, a heterogeneous viral population emerges. The rate of accumulation of mutations and evolution to virus with greater fitness depends on the viruses' replication rate (12). Since selective pressures differ between different tissue compartments and since the mucosal environment is characterized by higher concentrations of proinflammatory cytokines that may enhance HIV-1 replication, it is possible that the mucosa may harbor different quasiespecies than blood, playing a role in altered HIV-1 immunopathogenesis in the mucosa. Due to these evolutionary pressures, mucosal and peripheral blood viral quasiespecies conceivably may differ in their chemokine receptor tropism and replicative capacity. Different mutations

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(in the reverse transcriptase, protease, and envelope genes) have been described throughout patient's complement of CD4⁺ cells, in different compartments of the body, including the blood, brain, spleen, lymph nodes, and gastrointestinal tract (16, 32, 47). Compartmental differences in viral phenotype have also been described between virus isolated from the blood and lymph nodes (25). Al-Mulla et al. showed that virus isolated from the gastrointestinal mucosa and blood may differ phenotypically in terms of syncytium induction (2).

We sought to determine the factors involved in enhancing the susceptibility of the gastrointestinal mucosa to HIV-1 infection. We performed in vitro infections utilizing replication-competent R5-tropic HIV_{SX} and X4-tropic HIV_{NL4-3}, in which the *vpr* gene has been deleted and replaced with murine heat-stable antigen (mCD24). Upon viral replication in an infected cell, mCD24 is expressed on the cell surface and can be detected flow cytometrically. This allows for a determination of the percentage of cells that are infected with HIV-1, as well as for the phenotypic characterization of HIV-infected cells. Utilizing this approach, we show that a greater percentage of MMCs, compared to PBMCs, are infected by both M- and T-tropic viruses. Despite significant differences in chemokine receptor expression between cells in the gastrointestinal mucosa and blood, the cellular targets of the virus are fairly similar. R5- and X4-tropic HIV-1 predominantly target CXCR4⁺ CCR5⁺ cells in both compartments. Thus, the greater susceptibility of the mucosa to both viral phenotypes likely reflects the significantly greater presence of CCR5⁺ CXCR4⁺ cells in the mucosa than in the blood. Primary viral isolates obtained from the mucosal and blood compartments of the same HIV-1-infected patient exhibited similar chemokine receptor tropism. Consistent with the laboratory strains, infection of MMCs with these primary viral isolates resulted in greater p24 production than did infection of PBMCs. These data suggest that the greater ability of HIV-1 to infect and replicate within the gastrointestinal mucosa is secondary to differences in the cellular composition of these compartments rather than viral differences in chemokine receptor tropism or replicative capacity.

MATERIALS AND METHODS

Acquisition of MMCs and PBMCs. Rectosigmoid biopsies were obtained from healthy HIV-1-seronegative patients during routine colonoscopic examination being performed for colon cancer screening or diarrhea according to University of California at Los Angeles Institutional Review Board (UCLA IRB)-approved methods. Samples were acquired from HIV-1-infected patients who were recruited for a study utilizing mucosal anti-inflammatory agents to decrease mucosal HIV-1 replication according to UCLA IRB-approved methods. All samples were taken from HIV-1-infected patients at their baseline examination, prior to receiving study medication. Mucosal biopsies were routinely taken from a site 30-cm from the anus in the rectosigmoid colon to avoid potentially confounding inflammation resulting from traumatic or infectious proctitis. Biopsies were collected using large cup endoscopic biopsy forceps (Microvasive Radial Jaw #1589; outside diameter, 3.3 mm; Boston, Mass.) into 15 ml of RPMI (Irvine Scientific, Santa Ana, Calif.) with 10% fetal calf serum (FCS) (Gemini Bioproducts, Calabasas, Calif.) and supplemented with antibiotic-antimycotic solution (Gibco-BRL, Rockville, Md.) containing penicillin, streptomycin, and amphotericin B. The biopsies were maintained at room temperature on a rotating platform until isolation (20 to 60 min) and then moved to a 10-by-35-mm petri dish containing phosphate-buffered saline (PBS) in which the samples were teased apart using 18-gauge (18G) needles. The resulting isolated cells and minced tissues were resuspended in RPMI containing 10% FCS. While the use of minced biopsies precluded determination of the precise number of lympho-

cyte targets, we found that the mean yield of lymphocytes from four biopsies was $8.2 \times 10^5 \pm 2.5 \times 10^4$ ($n = 8$ donors).

Peripheral venous blood was collected from the patients in EDTA-containing tubes immediately prior to endoscopic acquisition of mucosal biopsies. PBMCs were prepared by centrifugation on a Ficoll-Hypaque density gradient (Nycomed Pharma AS, Oslo, Norway). An aliquot of 10^6 PBMCs was washed twice in RPMI and resuspended at a density of 10^6 cells/ml in RPMI supplemented with 10% FCS and 10 IU of interleukin-2 (IL-2; Amgen, Thousand Oaks, Calif.)/ml.

Reporter viruses. HIV-1 reporter constructs were made by cloning the cell surface molecule, murine heat-stable antigen (HSA; mCD24), into the partially deleted *vpr* gene region of the CXCR4-tropic strain, HIV-1_{NL4-3}, and the CCR5-tropic strain, HIV-1_{NFN-SX}. NL-r-HSAS (HIV_{NL4-3} expressing mCD24) was constructed as previously described (18, 19). To construct NFN-SX-HSAS (HIV_{SX} expressing mCD24), NL-r-HSAS was digested with *Pst*MI and *Eco*RI to liberate the *vpr*/HSA region. The 588-bp fragment was then ligated into NFN-SX (28) that had been previously digested with the same enzymes. In both cases, virus was prepared by transient transfection in 293T cells using calcium phosphate (37).

HIV-1 infection of biopsies and PBMCs. A total of 10^6 PBMCs were infected with 5×10^5 tissue culture infective doses (multiplicity of infection [MOI] of 0.5), and four minced biopsies were infected with an equivalent amount of virus, in a total volume of 1 ml for 4 h at 37°C. After incubation, the infected cells and biopsies were washed with 5 ml of PBS followed by 5 ml of RPMI and incubated in RPMI supplemented with 10% FCS and 10 IU of IL-2/ml. After 5 days in culture, MMCs were isolated from the minced biopsies by further tissue disruption achieved by sample passage through syringes with a series of decreasing needle gauges (18G to 21G). Debris was removed using a 70- μ m cell strainer (Becton Dickinson Labware, Franklin Lakes, N.J.). PBMCs were harvested from the plates using a cell scraper (Corning Inc, Corning, N.Y.). The single cell suspensions of isolated MMCs and PBMCs were analyzed by flow cytometry.

Flow cytometry. Monoclonal antibodies used in this study included CD3-allophycocyanin, CD4-phycoerythrin, CXCR4-allophycocyanin, and murine CD24 (all from PharMingen, San Diego, Calif.). Anti-CCR5 was provided by Walter Newman (Leukosite, Cambridge, Mass.) and was prepared as a 1:1 conjugate with phycoerythrin. Analysis was carried out on a FACSCalibur (BDIS, Mountain View, Calif.) with analysis using FACsExpress software (De Novo Software). Initial gating on the isolated MMCs and PBMCs was performed using side scatter and CD3 fluorescence. Dead cells were excluded from this population of lymphocytes by using 7-actinomycin (7-AAD; Calbiochem, San Diego, Calif.). The live T lymphocytes were then analyzed after gating on forward and side scatter. HIV-1-infected cells were identified by positive cell surface staining using anti-mouse CD24. By performing an additional gate on the murine CD24 cells, the expression of both CCR5 and CXCR4 receptors was ascertained on the infected cell population.

Isolation of primary isolates of HIV-1 from whole blood and mucosal biopsies. All HIV-1-infected patients who served as sources for blood and mucosal biopsies had CD4 cell counts of $>200/\mu$ l and HIV-1 plasma viral loads of $>5,000$ copies/ml despite the use of highly active antiretroviral therapy. Peripheral venous blood and four mucosal biopsies were collected from each patient as described above.

Phytohemagglutinin (PHA) blasts were prepared from PBMCs of healthy HIV-1-seronegative donors, resuspended at a density of 10^6 cells/ml in RPMI supplemented with 10% FCS and stimulated by 5 μ g of PHA (Sigma Chemical Corp, St. Louis, Mo.)/ml for 72 h. The cells were then washed three times with PBS, transferred to dishes containing 10^6 HIV-1 patient-derived PBMCs/ml or four mucosal biopsies, and then cultured in RPMI supplemented with 10% FCS and 10 IU of IL-2/ml. HIV-1 replication was assessed in this coculture system every 3 days. In each case, virus was used for infectivity studies from cocultures in which p24 levels were found to be >20 ng/ml on day 7. Virus-containing supernatant was sterile filtered and frozen at -80°C for future experimentation.

Assessment of replication of primary viral isolates. Mucosa- and PBMC-derived HIV-1, obtained by coculture, were used to infect MMCs and PBMCs of HIV-1-seronegative patients. Infection of 2.5×10^5 PBMCs or a single minced mucosal biopsy was carried out using 1 ml of primary viral isolates (p24 = 25 to 97 ng/ml). After incubation for 4 h, the infected cells and biopsies were washed in 5 ml of PBS and 5 ml of RPMI before being plated in a 24-well plate in 500 μ l of RPMI supplemented with 10% FCS, antibiotic-antimycotic solution, and 10 IU of IL-2/ml. Supernatants from these cultures were analyzed after 3 and 7 days for HIV-1 p24 protein by enzyme-linked immunosorbent assay (ELISA) (Coulter, Inc., Miami, Fla). Results are expressed in terms of nanograms of p24 produced per milliliter.

Chemokine receptor usage. Chemokine receptor usage of primary HIV-1 isolates was tested on human osteosarcoma cells stably transfected with human CD4 alone or with either CCR5 or CXCR4. Cotransfected into these cells is

green fluorescent protein (GFP) under the control of the HIV-1 long terminal repeat promoter. One milliliter of HIV-1-containing supernatant derived from cocultures of either the PBMCs or mucosa and 10 μ M Polybrene was added to the osteosarcoma cell line for 3 h. After the cells were washed twice, they were incubated for 48 h at 37°C and 5% CO₂. The viral tropism was determined by analyzing the cell lines flow cytometrically for expression of GFP, indicative of successful HIV-1 entry and replication.

Statistical methodology. Statistical comparisons were made between the percentage of MMCs and PBMCs that were infected with HIV-1, using a Student *t* test. All reported *P* values were found to be two-sided at the 0.05 significance level using Microsoft Excel software (Microsoft, Seattle, Wash.).

RESULTS

MMCs are more susceptible to infection with CCR5- and X4-tropic HIV-1 than are PBMCs. The high mucosal viral loads and selectively rapid depletion of mucosal CD4⁺ cells after SIV and HIV-1 infection suggest that the gastrointestinal mucosa represents a significant target for HIV-1. This may reflect enhanced HIV-1 replication among infected MMCs, due to the proinflammatory mucosal environment, and/or increased numbers of HIV-1-infected cells. Given the differences in the susceptibility of the mucosal and peripheral blood compartments to HIV infection, we hypothesize that the population of gastrointestinal mucosal cells are more prone to HIV infection than are PBMCs. We determined, by performing in vitro infections utilizing replication-competent R5-tropic HIV_{SX} and X4-tropic HIV_{NL4-3}, in which the *vpr* gene has been deleted and replaced with murine heat-stable antigen (mCD24), that a greater percentage of MMCs than PBMCs become HIV-1 infected. Upon viral replication in an infected cell, the mCD24 is expressed on the cell surface and can be detected flow cytometrically. This allows for a determination of the percentage of cells that are infected with HIV-1. Initial infections were conducted on a single cell suspension of MMCs obtained by enzymatic digestion, but rapid and extensive cell death made analysis difficult. Maintaining the mucosal environment by using mechanically disrupted biopsies, rather than a single cell suspension, and supplementation of the tissue culture medium with 10 IU of IL-2/ml permitted greater retrieval of live MMCs after a 5-day culture (data not shown). IL-2 was also added to PBMC cultures. Four biopsies taken with large-cup biopsy forceps will yield ca. 10⁶ MMCs, which were compared with infection of 10⁶ PBMCs.

After the 5-day infection, the disaggregated cells obtained from these biopsies contain a mixed population, including epithelial cells and stromal elements in addition to MMCs. In order to better examine infected mucosal T lymphocytes for the expression of mCD24, initial gating was performed using side scatter and CD3 fluorescence. Dead cells were excluded with 7-AAD, and live T lymphocytes were analyzed by gating on forward and side scatter. mCD24 expression was analyzed on CD3⁺ cells, since CD4 downregulation was notable.

As shown in Fig. 1, pairwise analysis of samples revealed that a significantly greater percentage of MMCs were infected with R5-tropic HIV_{SX} compared to PBMCs (2.6% $\pm$ 0.52% versus 0.74% $\pm$ 0.28% [mean $\pm$ standard error]; *P* < 0.005). This phenomenon was observed with each of the eight pairs of patient samples examined. Infection with the X4-tropic HIV_{NL4-3} also revealed a greater percentage of infected MMCs than PBMCs. In this case, 1.7% $\pm$ 0.29% of MMCs

were HIV-1 infected compared to 0.96% $\pm$ 0.05% of PBMCs (*P* = 0.04). In six of seven sets of samples studied, the percentage of infected MMCs exceeded the percentage of infected PBMCs as measured by mCD24 expression.

We conclude, based upon these results, that more MMCs are susceptible to both R5- and X4-tropic HIV-1, with a greater percentage of MMCs infected than PBMCs. Due to greater MMC cell death (29.16% $\pm$ 5.77% versus 8.72% $\pm$ 2.43%; *P* < 0.05), these results may actually underestimate the extent of the susceptibility differences between these compartments.

MMCs support more HIV replication per infected cell than do PBMCs. Once entry, reverse transcription, and integration have occurred, HIV-1 replication is dependent on the cell's transcriptional and translational machinery. HIV replication is enhanced in an immunologically activated lymphocyte. Therefore, the greater degree of cellular activation of gastrointestinal mucosal lymphocytes should result in more HIV-1 replication per infected cell. Comparison of the intensity of mCD24 expression permits relative quantitation of the amount of mCD24 on the cell surface, serving as a surrogate for the degree of HIV-1 expression supported by the cells.

After flow cytometric gating on the mCD24-expressing CD3⁺ cells, the mean fluorescence intensity of mCD24 was determined. Significant differences are noted when the mean fluorescence intensity values of mCD24 expression on infected MMCs are compared to expression on infected PBMCs after infection with HIV_{SX} and HIV_{NL4-3}. After infection with HIV_{SX}, the mean expression of mCD24 on MMCs was more than 10-fold higher than that seen on PBMCs (94.5% $\pm$ 9.8% versus 8.25% $\pm$ 0.95%; *P* = 0.002). The expression was lower after infection with HIV_{NL4-3}, although it was significantly higher on MMCs than PBMCs (70.8% $\pm$ 13.7% versus 7.6% $\pm$ 1.1%; *P* = 0.004). We conclude based upon these results that infected MMCs support greater R5- and X4-tropic HIV-1 replication than do infected PBMCs. This result supports the prior findings of greater activation of infected mucosal cells than PBMCs, although it may also reflect greater paracrine stimulation by proinflammatory soluble mediators.

The majority of MMCs coexpress CCR5 and CXCR4, while the majority of PBMCs express CXCR4 only. In order for HIV-1 to enter a CD4-bearing cell, binding to a second receptor must occur; CCR5 and CXCR4 are the principal second receptors used by HIV-1. The greater percentage of HIV_{SX}- and HIV_{NL4-3}-infected MMCs suggests that a larger percentage of MMCs may express CCR5 and CXCR4 than PBMCs. While previous studies have confirmed that the mucosa does harbor more CCR5-bearing cells, the percentages of CXCR4-bearing cells in the mucosal and peripheral blood compartments are similar. Since CCR5-expressing cells are predominantly of an activated/memory (CD26^{high} CD45RA^{low} CD45RO⁺) phenotype, while CXCR4⁺ CCR5⁻ cells are predominantly naive cells (CD26^{low} CD45RA⁺ CD45RO⁻), we hypothesized that the greater infection of MMCs by X4-tropic HIV_{NL4-3} might be due to coexpression of CCR5 (1, 5, 26). We have previously found that a greater percentage of CXCR4⁺ CD4⁺ cells in the mucosa are CD45RO⁺, similar to that seen among CCR5⁺ PBMCs (74 versus 46%; *P* < 0.05) (unpublished data). CCR5⁺ CXCR4⁺ MMCs should support greater HIV-1 replication than CXCR4⁺ CCR5⁻ cells. In order to

FIG. 1. Infection of PBMCs and minced biopsies with CCR5-tropic HIV_{SX} and X4-tropic HIV_{NL4-3} reporter viruses expressing mCD24. A total of 10^6 PBMCs, prepared by Ficoll-Hypaque density gradient centrifugation, were infected at an MOI of 0.5. In parallel, four biopsies obtained endoscopically from the rectosigmoid region of the same patient were minced and infected with an equivalent amount of virus for 4 h, washed, and cultured for 5 days in RPMI supplemented with 10 IU of IL-2/ml. Isolated MMCs and PBMCs were analyzed by flow cytometry. (A) Using side scatter and CD3 fluorescence, T lymphocytes were analyzed after gating on forward and side scatter. HIV-1-infected CD3⁺ cells were identified by positive cell surface staining by anti-mouse CD24. A higher percentage of MMCs than PBMCs were infected with HIV-1, as shown by the greater number of CD24⁺ (HIV-1-infected) cells extending rightward. (B and C) HIV_{SX} and HIV_{NL4-3} are the infected cell types (MMCs and PBMCs) on the x axis, and the percent CD3⁺ mCD24⁺ (HIV-1-infected) cells are on the y axis. A line is used to connect the percentage of infected PBMCs and MMCs from the same subject.

examine whether CXCR4⁺ MMCs and PBMCs coexpress CCR5, isolated cells were examined flow cytometrically for both chemokine receptors (Fig. 2). We confirm (3, 23) that the percentage of MMCs that expressed CCR5 was significantly greater than seen among the PBMCs ($69.9\% \pm 4.7\%$ versus $14.4\% \pm 2.8\%$; $P < 0.005$). Whereas 5.4% of MMCs were CCR5⁺ CXCR4⁺, 1.2% of PBMCs were CCR5 single positive ($P = 0.08$). In both the mucosal and peripheral blood compartments, >90% of cells expressed CXCR4, with no significant differences seen. On the other hand, significant differences were noted when coexpression of CCR5 was studied on these cells. Of all CXCR4⁺ PBMCs, $13.8\% \pm 7.6\%$ coexpressed CCR5 compared to $72.2\% \pm 11.2\%$ of CXCR4⁺ MMCs ($P < 0.005$).

Based upon these results we conclude that important differences exist between the mucosal and peripheral blood compartments in terms of cellular chemokine receptor expression. These differences in cellular phenotype might account for greater susceptibility of the mucosal compartment to both R5- and X4-tropic HIV-1. A significantly greater percentage of cells in the mucosal compartment than in the peripheral blood compartment express CCR5, permitting greater infection by HIV_{SX}. While >90% of mononuclear cells in each compart-

ment express CXCR4, significantly more CXCR4⁺ cells in the mucosal compartment coexpress CCR5, compared to cells in the peripheral blood.

R5- and X4-tropic HIV-1 predominantly infect CCR5⁺ CXCR4⁺ cells in the mucosal and peripheral blood compartment. The results presented above demonstrate that a greater percentage of MMCs than PBMCs are infected by HIV-1, a finding possibly explained by the greater percentage of CCR5⁺ CXCR4⁺ cells. We tested this possibility directly by examining the cell subpopulations infected by HIV-1 (Fig. 3). In order to characterize the cellular targets of the R5- and X4-tropic HIV-1 strains, we performed in vitro infections utilizing our reporter virus system. Given that the HIV-1 bears a reporter gene expressing mCD24, we are able to flow cytometrically characterize chemokine receptor expression on HIV-1-infected cells. The ability to characterize HIV-1-infected cells in this way represents the greatest strength of using the reporter virus constructs.

We found that a significantly greater percentage of MMCs, after infection with R5-tropic HIV_{SX}, are CCR5⁺ CXCR4⁺ ($15.8\% \pm 7.3\%$ versus $9.6\% \pm 6.9\%$; $P = 0.04$) compared to infected PBMCs. The majority of HIV_{SX}-infected cells in both compartments coexpressed CCR5 and CXCR4 ($75.4 \pm 7.2\%$

FIG. 2. CXCR4 and CCR5 expression on isolated PBMCs and MMCs. PBMCs prepared by Ficoll-Hypaque gradient and MMCs isolated from minced mucosal biopsies endoscopically obtained from the rectosigmoid region were studied flow cytometrically. CD45⁺ lymphocytes were analyzed after gating on forward and side scatter by staining for CXCR4 and CCR5. (A) Representative flow cytometry plots outlining chemokine receptor expression on PBMCs and MMCs. (B) Data compiled after analysis of 13 paired samples. The x axis outlines the chemokine receptor phenotypes (CXCR4⁺ CCR5⁻, CXCR4⁺ CCR5⁺, and CXCR4⁻ CCR5⁺), while the y axis specifies the percentage of the lymphocyte population with that phenotype. Error bars represent the means $\pm$ the standard error of the mean.

versus $65.3 \pm 5.8\%$; $P = 0.1$). The remainder of HIV_{SX}-infected cells (24.0% of infected PBMCs and 7.2% of infected MMCs) expressed CXCR4 but not CCR5. Since these cells were infected with R5-tropic virus, it is possible that the level

of surface expression of CCR5 was downregulated to undetectable levels, as has been previously described after HIV-1 infection (7). Similar to HIV_{SX}-infected cells, the majority of HIV_{NL4-3}-infected MMCs and PBMCs coexpress CCR5 and

FIG. 3. Chemokine receptor expression on HIV-1-infected cells. A total of 10^6 PBMCs, prepared by Ficoll-Hypaque density gradient centrifugation, were infected at an MOI of 0.5. In parallel, four biopsies obtained endoscopically from the rectosigmoid region were minced and infected with an equivalent amount of virus for 4 h, washed, and cultured for 5 days in RPMI supplemented with 10 IU of IL-2/ml. Isolated MMCs and PBMCs were analyzed by flow cytometry. T lymphocytes were analyzed, by using side scatter and CD3 fluorescence, after gating on forward and side scatter. HIV-1-infected CD3⁺ cells were identified by positive cell surface staining by anti-mouse CD24. The CD24-stained cells were then examined for expression of CCR5 and CXCR4. Graphs outlining the chemokine receptor expression on HIV-1-infected PBMCs and MMCs are shown for HIV_{SX} (A) and HIV_{NL4-3} (B). The x axis outlines the chemokine receptor phenotypes (CXCR4⁺ CCR5⁻, CXCR4⁺ CCR5⁺, and CXCR4⁻ CCR5⁺), while the y axis shows the percentage of infected CD3 lymphocytes with that phenotype. Error bars represent the means $\pm$ the standard error of the mean.

CXCR4 (PBMCs, $77.7\% \pm 3.3\%$; MMCs, $77.0\% \pm 5.8\%$; $P =$ not significant).

We conclude, based on these results, that despite significant differences in chemokine receptor expression between cells in the gastrointestinal mucosa and blood, the cellular targets of the virus are fairly similar. R5- and X4-tropic HIV-1 predominantly target CXCR4⁺ CCR5⁺ coexpressing cells in both compartments. Thus, the greater percentage of infected mucosal cells by both viral phenotypes likely reflects the significantly greater presence of CCR5⁺ CXCR4⁺ cells in the mucosa compared to the blood. Given the greater percentage of MMCs that express CCR5, it is likely that more MMCs than PBMCs are infected with R5-tropic virus. It is possible that an equal or greater number of PBMCs are infected with X4-tropic virus but are less able to support viral replication due to their low activation state.

Gastrointestinal MMCs support greater HIV-1 replication than do PBMCs. Due to the observed differences in the phenotype of MMCs and PBMCs, viral differences between the blood and mucosal compartments might also determine infectivity. We tested this possibility by examining the replication of primary isolates of HIV-1 cultured from paired mucosal biopsies and peripheral blood samples of HIV-1-seropositive patients. If mucosa-derived virus had increased replicative capacity compared to peripheral blood-derived virus, we would expect that the mucosa-derived virus would produce more p24 protein after infection of both MMCs and PBMCs. Alternatively, if cellular differences were accountable, as our results indicate then, regardless of the source of the infecting virus, greater HIV-1 replication would be seen after infection of MMCs than after infection of PBMCs. Infections of HIV-1-seronegative PBMCs and minced mucosal biopsies was performed with primary HIV-1 isolates obtained by short-term coculture from the blood and mucosal biopsies of HIV-1-infected patients. Primary viral isolates were obtained within 7 days of coculture to minimize selection of viral isolates with increased or decreased fitness.

As shown in Fig. 4, infections of minced mucosal biopsies using each of the three sets of mucosa- and blood-derived HIV-1 (patient 1, 97 ng of p24; patient 2, 25 ng of p24; patient 3, 75 ng of p24) produced increasing amounts of p24 protein over the 7 days of culture. In comparison, infection of PBMCs with these same sets of viral isolates showed either little or no increase in the p24 produced. As we observed above, the greater p24 production in MMCs compared to that seen in PBMCs likely reflects differences in chemokine receptor expression and the previously reported greater activation state of the mucosal cells (38, 49). These results support our hypothesis that cellular rather than viral differences account for the greater susceptibility of the mucosal compartment.

Tropism of paired viral isolates from blood and mucosal biopsies are similar. Differences in viral tropism may also play a role in the enhancement of HIV-1 infection of the gastrointestinal mucosa. For instance, the higher percentage of mucosal than peripheral blood cells bearing CCR5 might be expected to result in preferential selection for, and retention of, R5-tropic HIV-1 in the mucosa. In order to examine the effect of differences in viral tropism on observed cellular differences in HIV-1 infection, HIV-1 cultured from mucosal biopsies and peripheral blood was characterized in terms of its chemokine

FIG. 4. Comparison of replication of primary mucosa- and blood-derived viral isolates in MMCs and PBMCs. Primary HIV-1 isolates were cultured from PBMCs and four mucosal biopsies of HIV-1-infected subjects in the presence of PBMCs from HIV-1-seronegative donors stimulated by 5 μ g of PHA/ml. HIV-1 replication was assessed every 3 days. Virus-containing supernatants (patient 1, 97 ng of p24; patient 2, 25 ng of p24; patient 3, 75 ng of p24) were used to infect MMCs and PBMCs of HIV-1-seronegative patients. Supernatant from these cultures was analyzed after 3 and 7 days for HIV-1 p24 protein by ELISA. The results are expressed in terms of nanograms of p24 protein detected per milliliter. The results from infections using three pairs of mucosa (◆) and PBMC (△)-derived viral isolates are shown. The x axis outlines the day 3 and day 7 results of infections of MMCs and PBMCs, while the y axis shows the amount of p24 detected in the culture supernatant.

receptor tropism. Viral isolates were obtained after short-term coculture with activated PBMCs to minimize the selection of viral isolates with specific chemokine receptor tropism. We utilized an osteosarcoma cell line engineered to express human CD4 and either CXCR4 or CCR5 and which expresses GFP upon HIV-1 infection.

As shown in Fig. 5, virus derived from the blood and mucosa of these four HIV-1-seropositive patients showed qualitatively identical phenotypes. In patient 1, viral isolates from both compartments were capable of infecting both CXCR4- and CCR5-expressing cell lines. Virus derived from both the mucosa and blood of patient 2 was predominantly replicated in X4-bearing cells, while that from the blood and mucosa of both

FIG. 5. Comparison of chemokine receptor usage by primary mucosal- and blood-derived viral isolates. Primary HIV-1 isolates were cultured from the peripheral venous blood and four mucosal biopsies of HIV-1-infected subjects in the presence of PBMCs from HIV-1-seronegative donors stimulated by 5 μ g of PHA/ml. HIV-1 replication was assessed every 3 days. Virus-containing supernatants from the patients' mucosa and PBMCs were used to infect human osteosarcoma cells stably transfected with human CD4 alone or with either CCR5 or CXCR4. GFP was cotransfected into these cells under the control of the HIV-1 long terminal repeat promoter. The viral tropism was determined by analyzing the infected cell lines flow cytometrically for expression of GFP, a finding indicative of successful HIV-1 entry and replication. The flow cytometric plots derived from infections of CXCR4- and CCR5-expressing osteosarcoma cell lines using mucosa- and PBMC-derived primary HIV-1 isolates are shown. The plots show GFP expression on the x axis, while the y axis represents FL-2. GFP-positive cells are delineated by the R2 gate formed after the analysis of each viral isolate with a parental osteosarcoma cell line that was not transfected with a chemokine receptor.

patients 3 and 4 was predominantly R5-tropic. Based on these data, we conclude that HIV-1 found in the mucosa of these four patients does not differ from that found in the blood in terms of chemokine receptor utilization. Differences in viral tropism are unlikely to account for the differences in viral replication in these two compartments, and the mucosa does not appear to select for R5-tropic strains of HIV-1.

DISCUSSION

We undertook this study to examine the role of differences in chemokine receptor expression on infection of MMCs and PBMCs by R5- and X4-tropic HIV-1. A greater percentage of MMCs than PBMCs are infected by both R5- and X4-tropic HIV-1. Significant differences exist in terms of chemokine receptor expression in the blood and gastrointestinal mucosa; mucosal cells are predominantly CCR5⁺ CXCR4⁺, whereas

these cells make up less than 20% of the peripheral blood cells. This cell population is most susceptible to infection with both R5- and X4-tropic HIV-1 in both compartments. Regardless of whether viral isolates were derived from the blood or mucosa of HIV-1-infected patients, HIV-1 p24 production was greater in MMCs than in PBMCs. Further, the chemokine receptor tropism of these patient-derived viral isolates did not differ between compartments. We conclude based on these findings that the gastrointestinal mucosa represents a favored target for HIV-1, in part due to its large population of CXCR4⁺ CCR5⁺ target cells and not due to differences in the virus that it contains.

The gastrointestinal mucosa is a secondary lymphoid organ that contains the majority of the body's CD4⁺ lymphocyte population (6, 27) and appears to support enhanced HIV-1 replication compared to other body compartments (10, 24, 39). Our studies indicate that one significant reason for the unique

susceptibility of the gastrointestinal mucosal compartment is the greater infection of, and increased virus production from, CCR5⁺ CXCR4⁺ cells. We have shown that the majority of gastrointestinal mucosal CD4⁺ cells express both CXCR4 and CCR5 and are therefore susceptible to both R5- and X4-tropic HIV-1. In comparison, the majority of PBMCs are CXCR4⁺ CCR5⁻ and resistant to infection with R5 virus. The data we present in this study suggest that the unique susceptibility of the mucosal compartment is due, in part, to the greater percentage of CCR5⁺ CXCR4⁺ cells that it contains compared to blood. Still, we have not proven that mucosal CCR5⁺ CXCR4⁺ cells are more prone to R5- or X4-tropic HIV or produce more virus than do CCR5⁺ CXCR4⁺ PBMCs.

Antigenic stimulation of lymphocytes drives HIV replication (36, 40, 42). Therefore, secondary lymphoid organs are the main sites of HIV replication in vivo (8, 29). The majority of gastrointestinal mucosal T lymphocytes are memory cells and exhibit phenotypic and functional features of activation (31, 34). This likely reflects the vital immunologic role these cells play at the major boundary between humans and the external environment. The higher expression of CCR5 in the gastrointestinal mucosa likely reflects this state of inflammation since CCR5 is expressed on activated memory lymphocytes (5). Given these characteristics, the enhanced susceptibility to and replication of HIV-1 in the gastrointestinal mucosa is expected. Compared to PBMCs, the greater percentage of CCR5-expressing MMCs permits higher levels of infection by R5-tropic virus. Since these cells are predominantly of a memory and/or activated phenotype, they support HIV replication. Since the majority of MMCs expressing CXCR4 also coexpress CCR5 and are therefore memory and/or activated cells, infection of these cells with X4-tropic virus will also result in virus production. In comparison, infected CXCR4⁺ CCR5⁻ PBMCs, which have a naive phenotype, should not support significant viral replication. Since both M- and T-tropic HIV-1 predominantly infected CCR5⁺ CXCR4⁺ cells in both compartments, the higher expression of mCD24 on the infected MMCs may reflect a higher degree of activation in the mucosa of these phenotypically similar cells. We have previously shown that CCR5-bearing MMCs express higher amounts of CCR5 per cell than do CCR5-bearing PBMCs (3), which could potentially reflect a higher degree of activation.

A number of viral factors have been proposed to influence the clinical course of HIV-1 infection. These include quasispecies diversity, coreceptor usage, cellular tropism, and replicative capacity. HIV-1 quasispecies constantly evolve in response to their dynamic relationship with their cellular targets and the host immune response. If distinct viral evolution occurs in the PBMC and mucosal compartment, then viral factors could potentially explain the high mucosal susceptibility to HIV and SIV. Since viral replication and therefore genotypic evolution are driven by the inflammatory state of the infected cell, the dynamic inflammatory environment of the gastrointestinal mucosa may drive viral evolution to a greater extent than is seen in the peripheral blood compartment (41). Enhanced viral evolution at an inflamed mucosal site was suggested by Panther et al., who showed greater *env* heterogeneity in the female genital tract compared to paired blood samples (33). A number of other studies examining lymphoid and nonlymphoid tissue compartments have also suggested compartmentalization of

viral evolution (9, 15, 20, 21, 22, 30, 51). Though there have been few studies of HIV-1 evolution in the intestinal mucosa, genotypic differences in the *env*, *pro*, and reverse transcriptase genes of HIV-1 quasispecies in the intestinal mucosa and blood have been described (35, 43, 44). HIV-1 isolates from the gastrointestinal mucosa have also been shown to differ from paired blood isolates in terms of their ability to induce cytopathology in infected cells and showed a greater sensitivity to serum neutralization in one study (4). Although we did not find significant differences in the ability of mucosa- and PBMC-derived viruses to replicate in allogeneic MMCs or PBMCs, infection of MMCs supported greater replication of each viral isolate compared to infection of PBMCs. Therefore, cellular characteristics appeared to play a more vital role than did viral replicative ability.

Biological properties such as chemokine receptor tropism might differentiate mucosa-derived viruses from those isolated from other tissues and blood. Changes in the diversity of the viral envelope gene underlie changes in cellular tropism, coreceptor usage, and immune system evasion and therefore may determine the ability to infect and spread within cells in a given anatomic compartment (11, 17, 48). Compartmental differences in viral phenotype have been described in various tissues, including lymph nodes, spleen, bone marrow, kidney, liver, testes, lung, and brain (25, 45). Al-Mulla et al. showed that virus isolated from the gastrointestinal mucosa and blood may differ phenotypically in terms of syncytium induction (2), although these authors did not specifically examine chemokine receptor tropism. While most studies have suggested independent tissue-specific evolution, one study did show restricted sequence variability in the HIV *env* gene among different tissues that included the colonic mucosa. We found that chemokine receptor usage and tropism did not differ between viral isolates from the mucosal and PBMC compartments. This may be explained by the similar chemokine receptor expression of the cells that were infected in both compartments. In addition, in tissues, such as the gastrointestinal mucosa, where trafficking of lymphocytes is common, equalization of viral quasispecies between compartments may occur (13).

The results that we present may have important therapeutic implications. While the results of our in vitro assessments may not adequately mimic or reliably predict the biological complexity found in an HIV-infected person, these findings do suggest that further study of HIV immunopathogenesis in the mucosal compartment is necessary. Given the enhanced infection of mucosal lymphoid cells by HIV infection, perhaps therapies should be directed specifically toward this important compartment. Efforts to suppress mucosal inflammation could potentially decrease recruitment of CCR5⁺ CD4⁺ lymphocytes to the mucosa. Suppression of mucosal inflammation might decrease HIV replication in HIV-infected cells. Future work should further compare the activation state of the dual chemokine receptor-expressing cells in the blood and mucosa to determine whether a higher state of cellular activation accounts for the greater HIV-1 replication in MMCs. Additional experiments could also determine whether the CCR5⁺ CXCR4⁺ PBMCs home to, or derive from, the gastrointestinal mucosa.

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