Human Immunodeficiency Virus Type 1 Infection Is Associated with Significant Mucosal Inflammation Characterized by Increased Expression of CCR5, CXCR4, and β-Chemokines

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Mucosal inflammation is characterized by increased expression of proinflammatory cytokines and chemoattractant chemokines, resulting in infiltration of immunocompetent cells. This study compared the degree of mucosal inflammation in human immunodeficiency virus type 1 (HIV-1)-infected gut mucosa with that in tissue samples from subjects with inflammatory bowel disease (IBD) and from healthy seronegative control subjects. Gut mucosal biopsy specimens were immuno histochemically stained and were evaluated by in situ imaging. There was significantly increased expression of HIV-1 coreceptors CCR5 and CXCR4, β-chemokine RANTES, and macrophage inflammatory protein (MIP)–1α and MIP-1β, as well as increased numbers of T cells in lamina propria of HIV-1–infected patients. The results were similar in patients with IBD and in HIV-1–infected patients, suggesting increased inflammation in the colon of HIV–1–infected patients. To further investigate the effect of inflammation in HIV-1–infected lamina propria, treatments that reduce immune activation in lamina propria must be evaluated.

The gastrointestinal tract is a reservoir of a major pool of CD4+ T lymphocytes. Most of these cells are of the memory phenotype, expressing CD69 and CD45RO, and exhibit functional features of activation [1, 2]. In vitro studies have suggested that the differentiation of CD4+ T cells toward a memory phenotype is associated with increased susceptibility to human immunodeficiency virus type 1 (HIV-1) infection [3–6]. This finding supports the growing evidence that the HIV-1 burden is significantly higher in the intestine and in related lymphoid tissue than in peripheral blood [7]. A profound loss of CD4+ T cells in the intestine has been shown in simian immunodeficiency virus–infected macaques [8]. The gastrointestinal tract is also an important portal for entry and early dissemination of HIV-1 [9]. and numerous studies have identified HIV-1 in the intestinal mucosa [10, 11], even in patients with undetectable virus loads in plasma (authors’ unpublished data).

HIV-1 uses CD4 plus a coreceptor to infect cells. CXCR4 (CX chemokine receptor 4) and CCR5 (CC chemokine receptor 5) are the principal coreceptors for T cell–tropic/syncytium-inducing or X4 and macrophage-tropic/nonsyncytium-inducing or R5 HIV-1 strains, respectively [12–15]. CXCR4 is mainly expressed on naive CD4+ cells (CD45RA+), whereas CCR5 is predominantly expressed on memory CD4+ cells (CD45RO+) in healthy individuals [16]. Recent reports also have demonstrated enhanced CXCR4 levels on memory CD4+ lymphocytes [17].

In healthy HIV-1–seronegative persons, the expression of CCR5 is increased in mucosal mononuclear cells (MMC), compared with that in peripheral blood mononuclear cells (PBMC), whereas CXCR4 is expressed at similar levels on CD45RO+ T cells in MMC and PBMC [17]. It was recently shown that MMC are more easily infected with HIV-1 than are PBMC [17, 18]. Explanations for the high susceptibility of MMC to HIV-1 may include the increased expression of HIV-1 coreceptors, especially CCR5 [17], as well as the activation status of the resident cells [15, 19]. The expression of CCR5 is up-regulated by proinflammatory and Th1 cytokines, whereas Th2 cytokines up-regulate CXCR4 [20, 21]. This suggests that the expression of CCR5 and CXCR4 is partly controlled by Th1 or Th2 type cytokines, which are up-regulated in rectal mucosa from HIV-1–infected patients [22].

RANTES, macrophage inflammatory protein (MIP)–1α, and MIP-1β are the natural ligands for CCR5 [23], whereas stromal-

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derived factor-1 is the ligand for CXCR4 [24]. The physiologic function of β-chemokines and their receptors is to direct migration of recruited lymphocyte subsets to sites of inflammation and immune activation [25, 26]. Blocking of chemokine activity is effective for inhibiting the migration of certain leukocytes [27].

The up-regulation of chemokine receptors and of their ligands is a characteristic correlate of mucosal inflammation [28, 29]. Immune activation of resting CD4+ T cells triggers viral replication and dissemination [15, 19]. In this study, we investigated the expression of CCR5, CXCR4, RANTES, MIP-1α, and MIP-1β in colonic mucosal sites in HIV-1–infected patients and compared the results with those for healthy and inflammatory (subjects with inflammatory bowel disease [IBD]) control subjects. We quantified the levels of chemokines and chemokine receptors in cryopreserved, immunocytochemically stained tissues by in situ imaging and determined the phenotypic profile of isolated mucosal cells by use of flow cytometry. We investigated whether HIV-1 infection, similar to IBD, is associated with mucosal inflammation.

Materials and Methods

Patients. Rectosigmoidal biopsy specimens from 27 HIV–seropositive patients with plasma virus loads ranging from <40 copies/mL to 5.7 log10 copies/mL and CD4+ cell counts from 50 cells/mL to 967 cells/mL of blood were studied. All patients, except 1, were receiving antiretroviral treatment (table 1). Biopsy specimens from 8 active IBD control subjects were used as inflammatory controls. These 8 patients were symptomatic, despite treatment with anti-inflammatory drugs (table 1). Biopsy specimens from 14 healthy, HIV–1–seronegative subjects without organic bowel disease served as negative controls. From these patients, biopsy specimens from 6 HIV–1–infected patients, 5 inflammatory control subjects, and 5 healthy control subjects were analyzed by immunohistochemistry (table 1). The cryopreserved tissues were immunohistochemically stained for CD4, CD8, CCR5, CXCR4, RANTES, MIP-1α, and MIP-1β and were analyzed with in situ imaging. Rectosigmoid biopsy specimens from 24 HIV–1–seropositive patients, 3 inflammatory control subjects with IBD, and 9 healthy control subjects were analyzed by use of flow cytometry (table 1). Biopsy specimens from 3 patients in the HIV–1 cohort were analyzed by both methods. Isolated mucosal cells were stained for CD4, CD8, CD45, CCR5, and CXCR4 and were analyzed by flow cytometry.

Biopsy specimen acquisition and isolation of MMC. MMC were isolated from 4 endoscopic biopsy specimens from each donor, as reported elsewhere [17]. In brief, a standardized 30-cm site from the anus in the rectosigmoid colon was routinely sampled to avoid potentially confounding inflammation resulting from traumatic or infectious proctitis. Biopsy specimens were obtained by use of large-cup endoscopic biopsy forceps (Microvasal Radial Jaw no. 1589; Boston Scientific, Boston) with an outside diameter of 3.3 mm. Biopsy specimens for immunohistochemistry were immediately cryopreserved, and tissue samples for flow cytometry were placed in 15 mL of RPMI 1640 with 10% fetal calf serum. The biopsy specimens were maintained at room temperature on a rotating platform for 20–60 min before isolation. After rotation, the samples were removed to a 10 × 35 mm2 petri dish containing PBS with 1 mM EDTA and 50 mM 2-mercaptoethanol. The samples were teased apart using 18-gauge needles.

The disrupted tissue was incubated at 37°C for 20 min in a shaking water bath. After centrifugation, the tissue samples were digested with a mixture of collagenase and dispase (no. 269638; Boehringer-Mannheim, Mannheim, Germany; 0.1 mg/mL in RPMI) for 1 h at 37°C. Further tissue disruption was achieved by sample passage through syringes with a series of decreasing needle gauges (18G–21G). Debris was removed using a 70-µm cell strainer (Falcon no. 3520; Becton Dickinson Labware, Franklin Lakes, NJ). The resulting cells were resuspended in RPMI containing 10% fetal calf serum. MMC, which primarily include epithelial cells and leukocytes, were counted visually using a hemocytometer, and the proportion of mononuclear cells that were leukocytes was estimated. About 20% of the total MMC were leukocytes from a mean yield of 1.3 × 106 ± 1.1 × 106 (n = 6) from 4 biopsy specimens. Viability, as determined by the exclusion of trypan blue, was >90%.

Flow cytometry. Monoclonal antibodies (MAbs) used in this study included CD4–fluorescein isothiocyanate (clone SK3) and CD45–peridinin chlorophyll protein (clone 2D7; both from Becton Dickinson, San Jose, CA), CD8–allophycocyanin (clone SK1; Caltag Laboratories, Burlingame, CA), and anti–CXCR4-R-phycocerythrin (PE; clone 12G5; PharMingen, San Diego). Anti–CCR5 (clone 2D7) was provided by Walter Newman (Leukosite, Cambridge, MA) and was prepared as a 1:1 conjugate with PE by Kenneth Davis and Noel Warner (Becton Dickinson). A FACSCalibur flow cytometer was used with Cell Quest software (both from Becton Dickinson).

Initial gating on the isolated MMC was done using side-scatter and CD45 fluorescence. A well-defined and separate population of mucosal leukocytes was identified as CD45 hi b and CD45–peridinin chlorophyll protein (clone 2D1; both from Becton Dickinson, San Jose, CA), CD8–allophycocyanin (clone SK1; Caltag Laboratories, Burlingame, CA), and anti–CXCR4-R-phycocerythrin (PE; clone 12G5; PharMingen, San Diego). Anti–CCR5 (clone 2D7) was provided by Walter Newman (Leukosite, Cambridge, MA) and was prepared as a 1:1 conjugate with PE by Kenneth Davis and Noel Warner (Becton Dickinson). A FACSCalibur flow cytometer was used with Cell Quest software (both from Becton Dickinson).

Immunohistochemistry. Cryopreserved colon biopsy specimens embedded in OCT compound (Tissue-TEK, Mites, Elk hart, IN) were cut into 8 µm–thick sections, mounted on glass slides (HTC; Novakemi, Stockholm), and were fixed with 2% formaldehyde (Sigma, Stockholm). Slides were washed with balanced salt solution (Gibco, Paisley, UK) and were stored at −20°C until stained. The staining procedure, which was used to identify cell markers, HIV–1 coreceptors, and soluble mediators of inflammation at the single-cell level, has been described elsewhere [32, 33]. In brief, cells were permeabilized with 0.1% Saponin in balanced salt solution. After peroxidase quenching, blocking with 1% fetal calf serum, and avidin and biotin blocking, using a blocking kit (Vector Laboratories, Burlingame, CA), the sections were incubated overnight at room temperature with primary antibodies.
<table>
<thead>
<tr>
<th>Study subjects</th>
<th>CD4 cells/mL in blood</th>
<th>Plasma virus load</th>
<th>CDC classification*</th>
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<td>2000</td>
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**NOTE.** ND, not determined.
* Classification of severity of HIV disease, as suggested by CDC in 1993 [30].
+ Analyzed by in situ imaging.
+ Analyzed by flow cytometry.
+ All patients had ulcerative colitis, except patients I3 and I7 who had Crohn’s disease.
+ Patients N2, N3, and N5 had functional bowel symptoms.
After an additional blocking with serum, secondary, biotinylated antibodies were incubated at room temperature, followed by incubation with an avidin–biotin–horseradish peroxidase complex (Vectastain elite kit; Vector Laboratories). Color reaction was developed by 3-diaminobenzidine tetrahydrochloride (Vector Laboratories), and the tissue samples were counterstained with hematoxylin. The following antibodies were used: monoclonal CCR5 (mixture of M, 45S449.111; K, 45S31.111) and CXCR4 (44717.111); polyclonal affinity-purified, biotinylated RANTES (BAF 278); MIP-1α (BAF 270); MIP-1β (BAF 271; all from R&D Systems, Minneapolis); monoclonal CD4 (SK3) and CD8 (SK1; both from Becton Dickinson); secondary, biotinylated affinity-purified goat anti–mouse IgG1 and goat anti–mouse IgG2b (both from CalTag Laboratories); and donkey anti-goat (Jackson ImmunoResearch Laboratories, West Grove, PA). Control stainings, without the primary antibody, were done on the sections to control for nonspecific background. For antibodies with subtype IgG1 (CD4 and CD8), an irrelevant mouse IgG1 was used to control for subtype-specific nonspecific staining.

Quantification of chemokines, chemokine receptors, and cellularity by in situ imaging. Digital images of stained samples were transferred from a DMR-X microscope (Leica, Wetzlar, Germany) into a computerized image-analysis system (Quantimet 550 IW; Leica, Cambridge, UK), which allowed for the detection of 16.7 million different colors. Two different methods of analysis were used. The first method determined the percentage of positive area in the total area: 2–8 fields, depending on the size of the biopsy, with a total mean area of \(2.2 \times 10^4 \text{ mm}^2\), were assessed for positive-stained area and for the total area of hematoxylin-positive cells present. The intestinal epithelium, including the crypts, was excluded. The frequency of positively stained area was measured in a semiquantitative way by use of a specialized software program [33], and results were expressed as the percentage of positive area of total tissue area. Limiting dilution of cDNA-transfected cells injected into control tonsils indicated a sensitivity of the assay of \(>1\) positive cell per 1000 events [33]. This method was used for CCR5, CXCR4, RANTES, MIP-1α, and MIP-1β. The results achieved by this technique will reach lower percentages than the results achieved as the first method determined the percentage of positive area in the total area of hematoxylin-positive cells. In this method, the positive and negative stained part of the whole cell.

The second method of analysis determined the percentage of positive cells and cellularity. In this method, the positive and negative cells in the digital image were marked manually and were counted by a specialized in situ imaging computer program (Quantimet 550 IW; Leica, Cambridge, UK; program sequence used in these analyses made by Olia Norén). Results were given as the percentage of positive cells of all cells. The intestinal epithelium and colonic crypts were included from the area measured. This method was used for the quantification of CD4 and CD8 cells and of CCR5. Multiplying the frequency of positive cells by the number of cells per square millimeter generated the absolute number of CD4+ and CD8+ cells.

Statistical analysis. The Mann-Whitney U test was used for statistical analysis for comparison between study cohorts. Spearman rank correlations were used to measure the correlation of CD4 cell counts in blood and tissue. Comparisons yielding \(P < .05\) were considered significant.

Results

**CD8+ cells were significantly increased, and CD4+ cells remained unchanged in number in gut lamina propria of HIV-1-infected patients.** To define the degree of cellular inflammation characterized by the change in numbers of CD4+ and CD8+ cells in HIV-1-infected gut lamina propria, biopsy specimens were immunohistochemically stained for CD4 and CD8 cells and were evaluated by in situ imaging. We analyzed biopsy specimens from 6 HIV-1–seropositive patients, 5 seronegative healthy control subjects, and 5 seronegative inflammatory control subjects with IBD (table 1). The frequency of CD4+ cells was significantly increased in the lamina propria of inflammatory control subjects (47.9% ± 20.0%), compared with that in both HIV-1–infected patients (26.6% ± 5.7%) and healthy control subjects (23.3% ± 5.9%; \(P < .05\)). No statistical difference in frequency of CD4+ cells could be shown between HIV-1–infected patients and healthy control subjects.

There was a 4-fold mean increase in the number of CD8+ cells in the gut lamina propria of HIV-1–infected subjects (19.4% ± 13.0%), compared with that in healthy control subjects (4.6% ± 1.8%; \(P < .005\)). Inflammatory control subjects had a 2-fold increase in CD8+ cells (9.5% ± 3.4%), compared with the increase for healthy control subjects (\(P < .05\)). The HIV-1–infected patients showed a higher mean number of CD8+ cells than did inflammatory control subjects, but no statistical difference could be shown.

To evaluate the absolute cellular infiltration of the tissue, we analyzed the total number of cells per area by in situ imaging. The total cellularity in the lamina propria was presented as cells per square millimeter. The mean cellularity in inflammatory control subjects (11,430 ± 4140 cells/mm²) was \(\sim50\%\) higher than that for noninflammatory healthy control subjects (7130 ± 1412 cells/mm²). HIV-1–infected patients showed a mean nonsignificant increase of 20% (8565 ± 620 cells/mm²), compared with that of healthy control subjects. The absolute number of CD4+ plus CD8+ cells per area was calculated by multiplying the percentage of CD4+ plus CD8+ cells by the total number of cells per square millimeter, resulting in T cellularity. The mean T cellularity in HIV-infected patients (3990 ± 1687 cells/mm²) was increased \(\sim2\)-fold, compared with that in healthy control subjects (1990 ± 585 cells/mm²; \(P < .05\)). Inflammatory control subjects had a mean 3.5-fold increase (6898 ± 5030 cells/mm²), compared with that of healthy control subjects (\(P < .01\); figure 1). No statistical differences in the total T cellularity could be found between HIV-1–infected patients and inflammatory control subjects.

**CCR5 and CXCR4 expression was increased in the gut lamina propria of HIV-1–infected patients.** To quantify the expression of chemokine receptor expression in colon, we evaluated (by in situ imaging) biopsy specimens from 6 HIV-1–seropositive patients, 5 HIV-1–seronegative inflammatory control subjects with IBD, and 5 HIV-1–seronegative healthy control subjects (table 1). The expression of CCR5 in HIV-1–infected tissue reached a
Combined T cellularity in colonic mucosa in human immunodeficiency virus type 1 (HIV-1)-infected patients (mean, 3990 ± 1687), inflammatory bowel disease (IBD) control subjects (6898 ± 5030), and healthy control subjects (1990 ± 585), expressed as the number of T cells/mm². T cellularity was calculated by multiplying the total no. of cells/mm² with the percentage of cells positive for CD4 and CD8, as determined by in situ imaging. HIV-1-infected patients had a 2-fold increase, compared with that for healthy control subjects (P < .05, Mann Whitney U test), whereas IBD control subjects showed a mean 3.5-fold increase, compared with that for healthy control subjects (P < .01, Mann-Whitney U test). No statistical difference in total T cellularity was found between HIV-1–infected patients and IBD control subjects. Each symbol represents 1 patient.

The expression of CCR5 in inflammatory control subjects reached a mean of 9.1% ± 9.2% positively stained area of the total area, corresponding to a 2-fold increase, compared with that for healthy control subjects (P < .01). The expression of CCR5 in inflammatory control subjects reached a mean of 9.1% ± 9.2% positively stained area of the total area, corresponding to a 2-fold increase, compared with that for healthy control subjects (P < .01; figures 2 and 3A). Noninflammatory control subjects had a mean of 1.3% ± 1.2% positively stained area.

Additional computer-assisted manual counting analyses were done on the same samples to detect the percentage of positive cells of all lamina propria cells. The frequency of CCR5-expressing cells in HIV-1–infected tissue reached a mean of 63% ± 12.7% of all cells, corresponding to a 3-fold increase compared with that for healthy control subjects (P = .01). The frequency of CCR5-expressing cells in inflammatory control subjects reached a mean of 58% ± 34.2% of all cells, corresponding to a 2.5-fold increase, compared with that for healthy control subjects. Noninflammatory control subjects had a mean of 23% ± 13.3% positive cells (data not shown).

The expression of CXCR4 in HIV-1–infected tissue reached a mean of 8.5% ± 5.7% positive area of the total area, corresponding to a 9-fold increase compared with that for healthy control subjects (P < .01). The expression of CXCR4 in inflammatory control subjects reached a mean of 10.6% ± 11.1% positive area, corresponding to an 11-fold increase, compared with that for healthy control subjects (P < .05; figure 3B).

No significant correlation between CCR5 or CXCR4 expression and virus load could be shown (data not shown). However, the 2 HIV-1–infected patients with the highest expression levels of CCR5 and CXCR4 were the only patients with preserved CD4 cells and virus loads >2000 copies/mL (HIV-1–infected patients H1 and H5 in table 1).

Chemokine receptor expression in CD45⁺ T lymphocytes in colonic mucosa.

To characterize differences in the phenotypic expression profiles of the chemokine receptors CCR5 and CXCR4 on CD4⁺ and CD8⁺ cells in the colonic mucosa, we used flow cytometry to characterize isolated MMC from colon biopsy specimens from HIV-1–infected samples, healthy sero-negative control subjects, and inflammatory control subjects with IBD (table 1). Initial gating to separate the lymphocytes was done using side-scatter and panCD45 fluorescence. The frequency of CD4⁺, CD8⁺, CCR5⁺, and CXCR4⁺ cells was determined. Triple labeling was performed for CD45/CD4/CCR5, CD45/CD4/CXCR4, and CD45/CD4/CXCR4.

Flow cytometry assessment (HIV-1–infected patients H1, H3, H4, and H7–H15 in table 1) showed that the total frequency of CD45⁺ cells expressing CCR5 in gut lamina propria was similar in all 3 groups, with a mean of 57.4% ± 17.5% (range, 25%–92%) positive cells (data not shown). No significant correlation to virus load could be shown (data not shown). Triple labeling for CD45/CD4/CCR5 (all HIV-1–infected patients in table 1) showed that HIV-1–infected patients had a decrease in CD45⁺ CD4⁺ cells expressing CCR5 in the colonic mucosa, compared with that for both healthy control subjects and inflammatory control subjects (P < .05 for both). No significant correlation to virus load could be shown (data not shown). The mean incidence of CD45⁺ CD4⁺ cells expressing CCR5 was 32.0% ± 18.2% in HIV-1–infected patients, 65.9% ± 23.2% in inflammatory control subjects, and 53.6% ± 13.2% in healthy control subjects (figure 3C). In addition, there was a significant decrease in the mean number of CCR5 molecules/CD45⁺ CD4⁺ T cells in HIV-1–infected patients (3202 ± 1578 molecules/cell), compared with that in healthy control subjects (6854 ± 4182 molecules/cell; P < .01). The mean number of CCR5 molecules/CD45⁺ CD4⁺ cells in inflammatory control subjects was 3419 ± 1250 molecules/cell (figure 3D). In contrast to CCR5 expression on CD45⁺ CD4⁺ cells, no statistical difference could be shown between cells from HIV-1–infected biopsy specimens and healthy controls when evaluating CD45⁺ CD8⁺ cells expressing CCR5 (mean, 91.0% ± 16.3%; figure 3E) or CD45⁺ CD4⁺ cells expressing CXCR4 (mean, 67.4% ± 12.9%; data not shown). No significant correlation between CXCR4 expression and virus load could be shown (data not shown).

The total frequency of CD45⁺ CD4⁺ cells in HIV-1–infected individuals reached a mean of 12.7% ± 4.6%, corresponding to a 2-fold decrease compared with that for healthy control subjects (28.2% ± 4.6%: P < .0001). The frequency of CD45⁺ CD4⁺ cells in inflammatory control subjects reached a mean of 23.7% ± 19.7%, which was not significantly different from percentages seen in healthy control subjects (28.2% ± 4.6%). The frequency of CD4⁺ cells in the mucosa of HIV-1–infected individuals was correlated significantly with the CD4 cell count in...
Figure 2. Top, Micrograph illustrating immunohistochemical staining for CCR5 in cryopreserved colonic mucosa of human immunodeficiency virus type 1 (HIV-1)-infected patient. Positive area was stained brown by 3'-diaminobenzidine tetrahydrochloride (DAB); all cells were counterstained with hematoxylin. A, Crypts and epithelial cells were encircled (blue line) for exclusion, allowing analysis of the lamina propria area only. B, Detection of positively stained (yellow) area. C, Detection of the total cellular area (encircled with red lines). D, Composite graph of total cellular area (red) and CCR5-positive area (green) revealed. Results achieved from this field: total area measured was 22891.39 μm², cell area measured was 14858.98 μm², percentage of positive area in the total area was 11.01%. Bottom, Immunohistochemically stained CCR5-expressing cells (stained brown with DAB and counterstained with hematoxylin) in gut lamina propria from HIV-1–infected tissue sample (E), inflammatory bowel disease tissue sample (F), and healthy control tissue sample (G). Original magnification, ×220. Bar corresponds to 20 μm (E–G).
blood (P < .05, r = .406). The frequency of CD45^+CD8^+ cells in HIV-1–infected individuals reached a mean of 46.1% ± 15.6%, a significant increase compared with that for healthy control subjects (29.4% ± 10.2%; P < .05). The frequency of CD45^+CD8^+ cells in inflammatory control subjects reached a mean of 37.9% ± 22.1%, which was not statistically different from that for healthy control subjects.

The expression of RANTES, MIP-1α, and MIP-1β is increased in gut lamina propria from HIV-1–infected patients. To quantify the expression of the chemokine receptor ligands in colon, in situ imaging was performed on biopsy specimens from 6 HIV-1–seropositive patients, 5 HIV-1–seronegative inflammatory control subjects with IBD, and 5 HIV-1–seronegative healthy control subjects (table 1). β-Chemokine RANTES had a mean expression of 8.3% ± 3.6% positive lamina propria area in HIV-1–infected subjects, corresponding to a ~3-fold increase, compared with that for healthy control subjects (P < .05). The expression of RANTES in inflammatory control subjects showed a nonsignificant increase, compared with that for noninflammatory control subjects (P = .22), with a mean of 4.4% ± 0.7% (figure 4A). The mean RANTES expression in noninflammatory control subjects reached 2.6% ± 2.2%.

MIP-1α in HIV-1–infected subjects was expressed over a mean area of 1.0% ± 0.3%, corresponding to a ~3-fold increase, compared with that for healthy control subjects (P < .01; figure 4B). Inflammatory and healthy control subjects showed a mean expression of 0.5% ± 0.4% and 0.2% ± 0.2%, respectively.

MIP-1β–positive cells had a mean expression of 5.7% ± 1.6% positive lamina propria area in HIV-1–infected patients, corresponding to a ~30-fold increase, compared with that for healthy control subjects (P < .01). MIP-1β had a mean expression of 2.5% ± 1.3% in inflammatory control subjects, corresponding to a ~24-fold increase, compared with that in healthy control subjects (P < .01), and the healthy control subjects had a mean MIP-1β expression of 0.2% ± 0.2% (figure 4C).

Discussion

Here we showed that the total expression of both CCR5 and CXCR4 was significantly increased in HIV-1–infected lamina

Figure 3. Expression of human immunodeficiency virus type 1 (HIV-1) coreceptors in gut mucosa evaluated by in situ imaging of immunohistochemically stained biopsy specimens (A and B), and by flow cytometry (C–E). Results in A and B are expressed as percentage of positively stained area in the total area and in C–E as percentage of positively stained cells of the gated cells. A. Total expression of CCR5 in the gut lamina propria (mean expression in HIV-1–infected patients, 8.6% ± 6.3%; in inflammatory control subjects [inflammatory bowel disease, IBD], 9.1% ± 9.2%; and in healthy control subjects, 1.3% ± 1.2%). Statistical difference (Mann-Whitney U test) was achieved between HIV-1–infected patients and healthy control subjects (P < .01) and IBD control subjects and healthy control subjects (P < .05). Each symbol represents 1 patient. B. Total expression of CXCR4 in the lamina propria (mean expression in HIV-1–infected patients, 8.5% ± 5.7%; in IBD control subjects, 10.6% ± 11.1%; and in healthy control subjects, 0.9% ± 0.4%). Statistical difference (Mann-Whitney U test) was achieved between HIV-1–infected patients and healthy control subjects (P < .01) and IBD control subjects and healthy control subjects (P < .05). Each symbol represents 1 patient. C. Frequency of CD45^+CD4^+ cells expressing CCR5 (mean expression in HIV-1–infected patients, 32.0% ± 18.2%; in IBD control subjects, 65.9% ± 23.2%; and in healthy control subjects, 53.6% ± 13.2%). Statistical significance (Mann-Whitney U test) was achieved between HIV-1–infected patients and healthy control subjects and IBD control subjects (P < .05 for both). D. Mean number of CCR5 molecules/CD45^+CD4^+CCR5^+ cells for HIV-1–infected patients, IBD control subjects, and healthy control subjects. HIV-1–infected patients had a significant decrease (3202 ± 1578 molecules/cell), compared with that of healthy control subjects (6854 ± 4182 molecules/cell; P < .01, Mann-Whitney U test). Mean number of CCR5 molecules/CD45^+CD4^+CCR5^+ cells in IBD control subjects was 3419 ± 1250. E. Frequency of CD45^+CD8^+ cells expressing CCR5. There was no statistical difference among cohorts.
Figure 4.  Total expression of β-chemokines in lamina propria evaluated by in situ imaging on immunohistochemically stained biopsy sections. Results are expressed as percentage of positively stained area in the total area. A, RANTES (mean expression in HIV-1–infected patients, 8.3% ± 3.6%; in inflammatory control subjects [inflammatory bowel disease, IBD, 4.4% ± 0.7%; and in healthy control subjects, 2.6% ± 2.2%]. Significant difference (Mann-Whitney U test) was shown between HIV-1–infected patients and healthy control subjects (P < .05). Each symbol represents 1 patient. B, Macrophage inflammatory protein (MIP)-1α (mean expression in HIV-1–infected patients, 1.0% ± 0.3%; in IBD control subjects, 0.5% ± 0.4%; and in healthy control subjects, 0.2% ± 0.2%). A significant difference (Mann-Whitney U test) was shown between HIV-1–infected patients and healthy control subjects (P < .005). Each symbol represents 1 patient. C, MIP-1β (mean expression in HIV-1–infected patients, 5.7% ± 1.6%; in IBD control subjects, 2.5% ± 1.3%; and in healthy control subjects, 0.2% ± 0.2%). A significant difference (Mann-Whitney U test) was shown between HIV-1–infected patients and healthy control subjects (P < .005) and between IBD control subjects and healthy control subjects (P < .01). Each symbol represents 1 patient.

In this study, patients with plasma virus loads >2000 copies/mL and stable CD4 cell counts had the highest expression levels of CCR5 and CXCR4 in the gut lamina propria (HIV-1–infected patients H1 and H5 in table 1). This suggests that mucosal inflammation may facilitate viral replication and thereby worsen the prognosis for HIV-1–infected individuals. However, no differences in CCR5 expression frequencies on total lymphocytes (CD45bright) between HIV-1–infected patients and inflammatory and healthy control subjects could be detected by flow cytometry. Levels of CD4+ T cells expressing CCR5 (the most important targets for HIV-1) were decreased in HIV-1–infected patients, compared with that of healthy and inflammatory control subjects (P < .05 for both). An increased turnover of CD4+ and CD8+ cells has been suggested in HIV-1 infection [37–39]; however, there have been arguments against this suggestion [40, 41]. These results suggest a selective loss of the CD4+ T cells expressing CCR5 in HIV-1–infected lamina propria. Our findings of a selective loss of CD4+ CCR5+ T cells combined with the increased T cellularity would suggest an increased turnover of these cells at least within the intestinal mucosa.

There are several explanations for the apparent discrepancies between results obtained from in situ imaging and those obtained from flow cytometry. First, flow cytometry was performed on cells obtained from mechanically and chemically disrupted tissues, whereas in situ imaging was done on intact, cryopreserved, tissue samples. Computer-assisted manual counting of the immunohistochemically stained sections was done for CD4, CD8, and CCR5 cells. The results showed that the incidence of positive cells was much higher than the percentage of positive area. This difference is simply due to the fact that the whole cell surface of a positive cell is not stained. The relative difference observed among the study cohorts remained, however, significant.

In the 3 patients analyzed by both methods, the percentages of CD4+ and CCR5+ cells obtained by in situ imaging were higher than those obtained by flow cytometry, whereas frequencies for CD8+ cells were slightly lower. Immunohistochemistry and in situ imaging detect the total expression of these molecules in tissue, whereas only gated CD45+ cells were analyzed by flow cytometry. One explanation is that cells other than T cells, such as macrophages and dendritic cells, express CD4 and CCR5 molecules. This would give higher percentages by in situ imaging. Nevertheless, as long as the chemokines and chemokine receptors were expressed in the mucosa, they would still contribute to the inflammatory response. Another possible
explanation may be that preapoptotic T cells were lost during the isolation procedure before flow cytometric analysis. Furthermore, preapoptotic cells in HIV-1 samples may predominantly be the CD4+ cells expressing CCR5, which we showed are decreased in HIV-1–infected colonic mucosa. Immunohistochemistry can be considered as an optional way to analyze total expression, whereas flow cytometry is used for subgroup analyses.

Another explanation for the differences in expression of CD4, CD8, CCR5, and CXCR4 is that different patient materials were used for the 2 experiments. However, the similarities regarding plasma virus load and CD4 cell count between the cohorts strongly contradicts this explanation.

Because of different fixation protocols and the fact that none of the primary antibodies generated significant signals in both immunohistochemistry and flow cytometry, different primary antibodies had to be used with each technique. This may have contributed to some differences in results. The anti-CCR5 antibodies used for immunohistochemistry bind to the second loop of G-coupled proteins of the CCR5 molecule, which is affected neither by virus nor by chemokine binding. A high binding of virus or chemokines to the receptor might block binding of the anti-CCR5 antibody used in flow cytometry, causing false low results. In this study, the CCR5 density on CD45+CD4+ cells was decreased in HIV-1 patients, compared with that in healthy control subjects (P < .01), suggesting that high levels of β-chemokines or virus may have down-regulated CCR5 expression [18, 42, 43]. Nevertheless, the number of receptors per cell remains above the level identified for maximal infection in vitro [44].

Complex patterns of chemokine expression have been correlated with many human inflammatory diseases [25, 29, 45]. Triggering of inflammation in the upper respiratory tract caused increased levels of RANTES and MIP-1α [46, 47]. Here we showed that the expression levels of RANTES, MIP-1α, and MIP-1β were significantly increased in HIV–1–infected lamina propria, compared with that for healthy control subjects. Of note, the expression of β-chemokines was equal to, or even higher, in HIV-infected lamina propria than in active IBD samples. The strong increase of β-chemokines suggests that HIV-1, similar to IBD, is associated with increased mucosal inflammation and immune activation.

MIP-1α, MIP-1β, and especially RANTES inhibit HIV-1 infection in vitro [18, 42, 43]. Hence, high levels may indicate a protective effort against initial HIV-1 infection or spread. The initial trials that examined this were in vitro studies on isolated cells in suspension and with supraphysiologic levels of RANTES. One recent vaccine study reported protective correlation of low virus load and high levels of β-chemokines in plasma [48]. Recent studies addressing the in vivo function did not show any correlation between tissue virus load and the RANTES expression in lymphoid tissue in already infected macaques and humans [49, 50]. The high expression of RANTES and MIP-1β in HIV-infected tissue may rather, through its chemotactant effects, cause an increased influx of immunocompetent cells and result in activation of CD4+ and CD8+ T cells, increasing potential targets for HIV-1 infection and possibly apoptosis. This supports interpretations that increased levels, despite being a natural response to inflammatory stimuli, may enhance rather than prevent HIV-1 spread. In this study, all patients with a virus load >2000 copies/mL showed high levels of RANTES (HIV–1–infected patients H1, H5, and H6 in table 1), whereas the patients with lower virus loads had a broader spectrum of expression. This would suggest a correlation between high levels of RANTES and high plasma virus load, although our cohort was too small to assess this.

Increased levels of chemoattractant β-chemokines, soluble mediators of inflammation, would probably contribute to an increased cellular component of inflammation in the tissue. Increased cellularity is also a strong sign of immune activation in the gut mucosa. A significantly increased total cellularity was detected in inflammatory control subjects (P < .05). In HIV–1–infected tissue samples, we showed an increased mean cellularity that approached significance (P = .1), and, of importance, there was no reduction in cell numbers. Other reports have not revealed any difference in cellularity between HIV-1 patients receiving highly active antiretroviral therapy and in various disease stages and healthy control subjects [51]. However, when selectively evaluating the absolute number of T cells, we showed a significant increase in both HIV–1–infected patients (P < .05) and inflammatory control subjects (P < .01), compared with that in healthy control subjects, suggesting cellular inflammation in gut mucosa in HIV–1–infected individuals.

These results suggest that the increased levels of β-chemokines are attracting cells to the gut lamina propria in both HIV–1–infected patients and inflammatory control subjects. Since the CD8+ cells are increased and the CD4+ T cells are decreased or unchanged, a selective increased apoptosis among the CD4+ cells could be hypothesized [4, 37]. The most likely explanation for the overall observation is a combination of increased influx of total cells coupled with an increased turnover of CD4+ cells. This hypothesis is further supported by the fact that the frequency of CD45+CD4+ cells expressing CCR5, the most important targets for HIV–1, was selectively lower in HIV–1 patients than in inflammatory and healthy control subjects when evaluated by flow cytometry. Human gut mucosa may, as has been demonstrated in macaque models [8], be an important site for CD4+ T cell loss.

Here we showed that HIV–1 infection was associated with significantly increased expression of the chemoattractant chemokines (RANTES, MIP-1α, and MIP-1β), suggesting continuously increased soluble mediators of inflammation in the lamina propria of the colon. The increased T cellularity in HIV–1–infected patients shows that there is also cellular inflammation in the gut lamina propria. As in IBD, HIV–1 may cause mucosal inflammation. This inflammatory response may worsen the prognosis for HIV–1 patients by causing recruitment of additional potential
targets for HIV-1 infection and thereby contribute to the spread of HIV-1 and further CD4+ T cell loss. To further investigate this effect in HIV-1 mucosal pathogenesis, therapies that reduce inflammation in the intestinal mucosa must be evaluated.

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