Enhanced levels of functional HIV-1 co-receptors on human mucosal T cells demonstrated using intestinal biopsy tissue

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Objective: To examine compartmental differences in co-receptor expression on CD4 lymphocytes between blood and gut using endoscopic biopsies.

Design: Mucosal and peripheral CD4 T cells from healthy controls were compared for co-receptor expression and vulnerability to infection by HIV-1.

Methods: Expression of CCR5 and CXCR4 was quantified by flow cytometry on isolated mucosal CD4 lymphocytes obtained from endoscopic biopsies and blood from healthy controls. Vulnerability to in vitro infection by both R5 and X4 strains was assessed by measuring p24.

Results: Biopsies yielded sufficient lymphocytes for flow cytometric characterization and infectivity studies. The percentage of mucosal CD4 T lymphocytes that expressed CCR5 and the per cell expression of CCR5 were both significantly increased compared with that in peripheral blood CD4 T lymphocytes. CXCR4 was expressed on the majority of CD4 lymphocytes in both compartments. In vitro infection of mucosal mononuclear cells supported greater viral replication of both R5 and X4 strains than peripheral blood mononuclear cells.

Conclusions: Enhanced expression of CXCR4 and CCR5 on CD4 lymphocytes in normal intestinal mucosa predicts increased vulnerability to infection by both R5 and X4 HIV-1. Endoscopic biopsies provide a useful mucosal tissue sampling technique to identify compartmental immunologic differences that may be exploited by HIV-1 in establishing initial mucosal infection.

AIDS 2000, 14:1761–1765

Keywords: human, gastrointestinal, mucosal, HIV-1 co-receptors, CCR5, CXCR4, HIV-1 biopsy

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Date of receipt: 6 September 1999; revised: 3 March 2000; accepted: 28 April 2000.
Introduction

The majority of HIV-1 transmission occurs across mucosal surfaces. The mechanisms associated with transmission are complex, but the local mucosal immune system is likely to play a critical role in susceptibility to, or protection from, HIV-1 infection. The normal intestinal mucosa is characterized by mild inflammation maintained by constitutive expression of locally secreted chemokines and cytokines [1–3]. Further, gastrointestinal lymphocytes differ functionally and phenotypically from their peripheral blood counterparts [4–6]. Virtually all mucosal CD4 lymphocytes express activation markers and are of the CD45RO memory subset [4–7].

The vulnerability of intestinal mucosa to HIV-1 infection following sexual exposure or ingestion of milk from HIV-1-infected mothers is well recognized, but the mechanisms remain unclear. Using surgically resected specimens, Lapenta et al. demonstrated an increased expression of CCR5 on human mucosal CD4 T cells compared with peripheral blood mononuclear cells (PBMC) [8]. This increase in co-receptor expression might augment mucosal vulnerability to HIV-1 infection and might represent a potential target for viral blockade [9,10].

These results confirm the importance of the mucosa in the pathogenesis of HIV-1 disease and the role for convenient and repetitive methods to sample mucosal tissue using endoscopic biopsies. In this study, isolated mucosal mononuclear cells (MMC) were obtained from mucosal biopsy samples and co-receptor expression on CD4 cells quantified by flow cytometry. The susceptibility of MMC and PBMC to infection by both R5 and X4 strains of HIV-1 was investigated.

Methods

Study subjects and endoscopy
Intestinal tissue was collected from eight healthy HIV-negative individuals undergoing elective endoscopy for a history of blood in stool or for routine polyp screening. No subjects had diarrheal symptoms or history of inflammatory or infectious intestinal disorders. Biopsies were acquired from a standardized site of 30 cm in the rectosigmoid colon to avoid potential regional variation. All samples were histopathologically normal. Phlebotomy was performed immediately preceding endoscopy; blood was collected in ethylenediamine tetracetic acid (EDTA)-containing tubes. Informed consent was obtained from all patients and the study was approved by the UCLA Human Subjects Protection Committee.

Biopsy acquisition and isolation of mucosal mononuclear cells
MMC were isolated from four endoscopic biopsies from each donor and were placed into 15 ml of tissue culture medium (RPMI 1640, Irvine Scientific, Santa Ana, California, USA). The biopsies were maintained at room temperature on a rotating platform until isolation (20–60 min), then removed to a 10 × 35 mm petri-dish containing phosphate-buffered saline (PBS) with 1 mmol/l EDTA and 50 mmol/l 2- mercaptoethanol and teased apart using 18G needles. The disrupted tissue was incubated at 37°C for 20 min in a shaking water bath. Following centrifugation, the tissue samples were digested with a mixture of collagenase and dispase (0.1 mg/ml in RPMI; Roche Molecular Biochemicals, Indianapolis, Indiana, USA) for 1 h at 37°C. Further tissue disruption was achieved by sample passage through syringes with a series of decreasing needle gauges (18G to 21G). Debris was removed using a 70-μm cell strainer (Falcon; Becton Dickinson, Franklin Lakes, New Jersey, USA). Isolated cells were resuspended in RPMI containing 10% fetal calf serum. MMC, which includes primarily epithelial cells and leukocytes, were counted visually using a hemocytometer and the proportion of mononuclear cells that were leukocytes estimated. Approximately 20% of the total MMC were leukocytes from a mean yield of 1.3 × 10^6 cells (SD 1.1; n = 6) per four biopsies. Viability, determined by the exclusion of trypan blue, was > 90%.

Flow cytometry
Monoclonal antibodies included CD4-fluorescein isothiocyanate and CD45-peridin chlorophyl protein (BDIS, Mountain View, California, USA), CD8-allophyocyanin (Caltag, Burlingame, California, USA), and anti-CCR4-R-phycocerythrin (PE; Pharmingen, San Diego, California, USA). Anti-CCR5 was provided by Dr Walter Newman of Leukosite, (Cambridge, Massachusetts, USA) and was prepared as a 1:1 conjugate with PE. Analysis was carried out on a FACSCalibur (BDIS) and Cell Quest (BDIS) software. Initial gating on stained PBMC from whole blood and the isolated MMC was performed using side scatter and CD45 fluorescence followed by forward- and side-scatter gating. A well-defined and separate population of mucosal leukocytes was identified as CD45_{bright}, representing 10–50% of the initial mononuclear sample population. Of these, 20–40% were CD4 lymphocytes and 26–41% were CD8 T cells.

PBMC, isolated and directly analyzed by flow cytometry, showed no discernible differences in phenotypic profiles for all surface antigens when compared with PBMC processed through the mucosal isolation procedure (collagenase/dispase treatment) and then stained (data not shown).
To estimate the number of CCR5 molecules per CD4 lymphocyte, the observed CCR5 relative fluorescence intensity (RFI) was multiplied by a calibration factor, specifically 44, determined for our FACSCalibur. This calibration factor is the number of molecules of PE detected per RFI channel number. For monoclonal antibody prepared as 1:1 conjugates with PE, such as our anti-CCR5, the RFI channel number can be multiplied by the calibration factor to estimate the number of antibody molecules bound per cell [11]. This calculation was not performed for CXCR4 as it was not available as a 1:1 conjugate.

In vitro infection studies
MMC and PBMC were assessed for in vitro HIV-1 infection. Isolated MMC were cultured for 3 days in Iscove's DMEM medium (Gibco BRL, Rockville, Maryland, USA) supplemented with 10% human serum, containing 10 μg/ml gentamycin, penicillin, streptomycin (Gibco) and glutamine. Interleukin 2 (IL-2; Amgen, Thousand Oaks, California, USA) was added at 20 IU/ml. A total of $1 \times 10^5$ MMC and $1 \times 10^5$ PBMC were plated in a 96-well plate in 100 μl medium after a 3-h infection with HIV-1 SX or HIV-1NL4-3 at an multiplicity of infection of 0.01. Prior to plating, the cells were washed twice to remove free virus and adherent p24. Following plating, 30 μl supernatant was sampled at 18, 72 and 130 h for estimation of p24 production (per $10^4$ CD4 lymphocytes) using enzyme-linked immunosorbent assay (ELISA; Coulter Corp., Miami, Florida, USA). CD4 percentages were determined by flow cytometry and were used to determine the number of CD4 lymphocytes in the cultures.

IL-2 was required to maintain viability of mucosal cell populations. As IL-2 is known to upregulate CCR5 and could enhance viral replication [12,13], PBMC from the same patient were exposed to infectious virus both with and without IL-2 present in the culture media to control for the IL-2 effect.

Statistical methodology
Two-sided Wilcoxon signed rank tests were used to examine difference in phenotypic marker values between blood and gut samples; $P$ values $\leq 0.05$ were considered statistically significant.

Fig. 1. CCR5 receptor expression on CD4 lymphocytes from blood and from gut mucosa. (a) The percentage of CCR5-expression of CD4 lymphocytes from blood and gut of six subjects are shown accompanied by the inset flow cytometry scatter plots (CCR5 and CD4 staining) for blood and gut of a representative subject. The number on the upper right quadrant of each inset plot indicates the percentage of CD4 lymphocytes in that subject’s compartments (blood or gut) that expressed CCR5. The gut samples of all six subjects had a greater percentage of CCR5$^+$CD4$^+$ cells compared with blood ($P = 0.03$); differences ranged from 2.0-fold to 5.4-fold. (b) The number of CCR5 receptors per cell on blood and gut CD4 lymphocytes of the same six subjects are shown accompanied by flow cytometry histograms of a representative subject. The rightward shift of mean fluorescence index in the CCR5$^+$CD4$^+$ mucosal cells in the inset histogram illustrates the increased numbers of CCR5 receptors per CD4 lymphocyte, while the numbers above the bars indicates the number of molecules of CCR5 expressed per CCR5$^+$CD4$^+$ lymphocyte in the blood and gut of that individual. The gut samples of all six subjects had higher expression of CCR5 molecules per cell compared with the blood ($P = 0.03$); differences ranged from 1.4-fold to 3.5-fold. Symbols for each person are the same as those used in (a).
Results

Co-receptor expression on mucosal and blood CD4 T cells
A median of 23% [interquartile range (IQR) 18–30] of all blood CD4 lymphocytes expressed CCR5. A median of 71% (IQR 50–87) of the mucosal CD4 lymphocytes expressed CCR5, a 2.8-fold increase over those in blood (P = 0.03) (Fig. 1a). Mucosal CD4 lymphocytes also expressed significantly more (2.2-fold increase) CCR5 receptors per cell (6946 molecules; IQR 6306–10 416) than did their CCR5-expressing CD4 lymphocyte blood counterparts (3841 molecules IQR 3259–4441) (P = 0.03) (Fig. 1b). Taken together, this is a 6.2-fold increase in total mucosal expression of CCR5 receptors on CD4 lymphocytes potentially available for viral infection compared with that in the blood.

Nearly all (97%) of the detected CCR5 expression on CD4 lymphocytes in both the blood and gut was on memory (CD45RO) cells. There was an increased proportion of CD45RO memory cells among gut CD4 lymphocytes (median 95%; IQR 90–97) compared with blood (median 46%; IQR 38–53). While not all memory cells expressed detectable levels of CCR5, nearly all CCR5 was on the preferentially infectable memory T cells, in both compartments.

In contrast to the findings with CCR5, high levels of CXCR4-expressing cells were observed in both blood (median 83%; IQR 75–87) and gut (median 64%; IQR 59–79) without significant difference between the two compartments either in percentage or in relative fluorescence intensity of staining with anti-CXCR4 antibody (data not shown). Based on co-receptor expression, mucosal lymphocytes would likely be infectable by HIV-1 strains with tropism for either co-receptor [14].

In vitro infection studies of MMC compared with PBMC
To contrast the susceptibility to HIV-1 infection of MMC with enhanced co-receptor expression with that of PBMC and to demonstrate the functional relevance, both cellular populations were subjected to in vitro HIV-1 infection. As shown in Fig. 2, MMC were able to support vigorous viral replication in culture compared with that achieved with PBMC, with or without IL-2. PBMC infected in the absence of IL-2 could not support HIV-1 replication by either HIV-1SX or HIV-1NL4-3. When compared with similarly cultured PBMC in the presence of IL-2, MMC produced markedly more p24 following infection with R5 and X4 isolates of HIV-1. At 72 h, supernatant p24 levels of the R5 HIV-1SX in the MMC culture was 164 pg/ml per 10^4 CD4 lymphocytes compared with 51 pg/ml per 10^4 CD4 cells in the PBMC cultured with IL-2. For X4 HIV-1NL4-3, viral growth accelerated over time and supernatant p24 levels in the MMC cultures at 130 h was 1194 pg/ml per 10^4 CD4 lymphocytes compared with undetectable levels in cultures of PBMC.

Discussion

Endoscopically acquired intestinal mucosal biopsies can be used to isolate of MMC in sufficient quantities to perform flow cytometric characterization of this compartment and to undertake in vitro infection studies.

Our results show that there is enhanced expression of the chemokine receptor CCR5 on MMC compared with that on PBMC. Moreover, both the proportions of mucosal CD4 cells expressing CCR5 and the amount of CCR5 expressed per cell were increased. In
contrast to previous reports, we found significant CXCR4 expression on both naive (CD45RO−) and memory (CD45RO+) mucosal CD4 T cells [11,15]. Finally, we have demonstrated that these enhanced levels of co-receptor expression are functionally correlated with a far greater level of productive infection of MMC than PBMC, using both R5 and X4 HIV-1 isolates. CCR5 is the co-receptor most associated with HIV-1 in early infection and the gastrointestinal tract is the most common route of transmission [16]. Hence, the amount of detected CCR5 expression carries important implications for transmission, primary infection, local spread and treatment.

Although we did not detect compartmental differences in the CD4 T cell expression of CXCR4, high level of expression of this co-receptor in both compartments was observed. The enhanced vulnerability of mucosal lymphocytes to replication of both R5 and X4 strains of HIV-1 compared with PBMC is notable and requires further attention to explain the detection primarily of R5 virus in early infection when tropism and susceptibility to X4 virus is also present [17–19]. Mucosal T cells appear to provide a rich milieu for replication of variants of HIV-1 that use either of the major co-receptors for entry because of their high expression of the co-receptors on memory CD4 T lymphocytes and the high level of cellular activation [15,20,21].

Acknowledgements

We thank W. Newman for providing the anti-CCR5 antibody, K. Davis and N. Warner for preparing it as a 1:1 conjugate, and P. Hultin for technical assistance.

References