Effect of Genital Ulcer Disease on HIV-1 Coreceptor Expression in the Female Genital Tract

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Objective. To examine the expression of human immunodeficiency virus type 1 (HIV-1) coreceptors (CCR5 and CXCR4) by monocytic cells within human genital ulcers.

Methods. Women with primary or secondary syphilis, herpes simplex virus type 1 (HSV-1) or HSV-2 infection, or noninfectious abrasions had a biopsy sample taken from the lesion and contralateral vulva. HIV-1 coreceptor expression on CD3+ and CD14+ cells was analyzed by flow cytometry. Real-time reverse-transcriptase polymerase chain reaction was used to assess levels of coreceptor mRNA expression.

Results. Women with primary or secondary syphilis or with HSV-1 or HSV-2 infection had significantly increased numbers of CD14+ cells expressing CCR5 within the genital ulcer. This increase was also noted in the nonulcerated tissue isolated from women with syphilis and in peripheral blood mononuclear cells from women with secondary syphilis. CCR5 mRNA expression was increased in tissue obtained from syphilis lesions.

Conclusions. Monocytes recruited to genital ulcer disease (GUD) sites express increased levels of CCR5. This increased expression could account, at least in part, for enhanced HIV-1 transmission in the setting of GUD.

There is now compelling evidence that genital ulcer disease (GUD) is a significant risk factor for the bidirectional transmission of HIV-1 [1–8]. This epidemiologic interrelationship is, in part, predicated on the high-risk behaviors that facilitate transmission of the etiologic agents responsible for both GUD and HIV-1. However, a number of other biological and molecular factors also likely account for a large percentage of the increased risk for both acquisition and enhanced infectiousness of HIV in the setting of GUD. Physical disruption of the protective epithelial/mucosal barrier seems to be one important component contributing to the increased risk of both acquisition and transmission of HIV-1. In addition, the resulting inflammatory cellular milieu associated with GUD, usually consisting of macrophages, activated lymphocytes, and dendritic cells, also ostensibly facilitates HIV-1 acquisition by providing a rich source of immune cells expressing the relevant receptors for the virus [3, 9–13]. Consistent with this, cocultures of various GUD agents with HIV-1–infected cells give rise to enhanced HIV replication in vitro [14]. Additional in vitro studies also have provided strong evidence of HIV-1 coreceptor up-regulation after stimulation with Treponema pallidum subspecies pallidum (T. pallidum) or synthetic lipopeptide analogues modeled after its proinflammatory membrane lipoproteins [14–17]. There is, however, a paucity of in vivo–derived data corroborating many of the molecular findings garnered using in vitro model systems. We therefore designed the present study to assess the effect of GUD on HIV-1 coreceptor expression in the natural setting. To accomplish this, immune cells harvested either systemically (circulation) or locally (ulcer site) from patients with various GUDs were examined for cell-surface expression of HIV-1 coreceptors. HIV-1 coreceptor expression also was assessed at the mRNA level.

SUBJECTS, MATERIALS, AND METHODS

Human subjects. Healthy, sexually active female volunteers without a history of HIV infection who presented with a genital ulcer, with a noninfected vulvovaginal abrasion (to evaluate the effect of nonspecific inflammation), or without lesions (negative control
cells were treated with FACS Lysing Solution (BD Biosciences) and stained. CD14 is a cell marker found predominantly on monocytes and macrophages and acts as a receptor for bacterial lipopolysaccharide (LPS) and LPS-binding protein in serum. CD3 is expressed specifically on T cells and is associated with the T cell antigen receptor (TCR). After 30 min of incubation on ice, the cells were washed twice with PBSA + EDTA and then centrifuged at 900 g for 10 min. The cells were washed twice with PBSA + EDTA and stored at 4°C in 250 µL of 2% paraformaldehyde for flow cytometry.

**Tissue biopsy samples.** A 5-mm punch biopsy sample was obtained from the vaginal introitus of women lacking lesions (negative control subjects) or from ulcer sites or abrasion. A second biopsy sample was taken from uninfected genital tissue from the contralateral side. The biopsy samples were immediately divided into 2 equal portions and processed within minutes after collection for both immune cell isolation (for FACS analysis) and RNA isolation (to be used in chemokine receptor mRNA quantification).

The biopsy portion for FACS analysis was again divided into 2 sections and placed in RPMI 1640 medium supplemented with 10% human serum. These sections were washed 3 times. Each biopsy sample was minced in a small Petri dish containing 1 mL of RPMI 1640 medium. The material was incubated with collagenase (Roche) and DNAse (Sigma) for 90 min at 37°C, filtered through a 70-µm mesh filter, and washed with PBSA + EDTA. The subsequent procedure was the same as outlined above for mononucleocyte preparations.

**Flow cytometry.** The fluorochrome-conjugated erythrocyte-depleted leukocytes and the isotype-matched controls were then analyzed in duplicate using a FACSCalibur dual-laser flow cytometer (BDIS) with appropriate forward- and side-scatter gates to exclude debris, dead cells, and residual erythrocytes. A minimum of 100,000 events were assessed for each staining—this was achieved for all of the peripheral blood samples and for the majority of the tissue biopsy samples. Thirty-one percent of the tissue biopsy samples (all from the negative control subjects) did not reach 100,000 events, although all achieved ≥20,000 events in duplicate. Paint-A-Gate Pro software (BDIS) was used for data analysis.

**RNA isolation.** To isolate RNA from whole blood, the RiboPure Blood Kit (Ambion) was used in accordance with the manufacturer’s instructions. To isolate RNA from the tissue samples, an RNAqueous Kit (Ambion) was used. The tissue was homogenized in the kit’s lysis/buffer solution in a glass homogenizer. Total RNA was eluted into 100 µL of elution buffer and supplemented with 5 U of recombinant RNasin (Promega). RNA quality was assessed by determining individual 260/280 ratios. RNA quantification was performed using a NanoDrop spectrophotometer (NanoDrop Technologies) with a detection limit of 2 ng/µL. The samples were stored at −80°C.

The samples were treated with DNAase (DNA-Free; Ambion) at 37°C for 1 h to remove contaminating DNA and then quantified with the NanoDrop spectrophotometer. To verify the absence of contaminating DNA, 60 ng of the sample, positive control human DNA, and a reagent blank were amplified separately in 40 cycles of a PCR using β-actin forward primer (5'-GGCATCCTCACCCCTGAAGTA-3') and β-actin reverse primer (5'-GGGGTGGTGAAGGTCTCAAA-3') [22]. The cycling con-
conditions were TaqMan assay conditions (95°C for 15 s and 60°C for 1 min).

**cDNA synthesis and real-time quantitative reverse-transcriptase PCR (qRT-PCR).** The High Capacity cDNA Archive Kit (Applied Biosystems) was used to enable the quantitative conversion of total RNA to single-stranded cDNA using a master cycler gradient. The total 50–µL cDNA reaction consisted of 2 µg of RNA, brought up to a nucleic-free water volume of 25 µL. To this was added a master mix containing 5 µL of 10× RT buffer, 2 µL of 25× dNTP mix, 5 µL of 10× random primers, 2.5 µL of 50 U/µL RT, and 10.5 µL of nuclease-free water. The reaction mixture was incubated at 25°C for 10 min and at 37°C for 2 h.

qRT-PCR was performed on blood and tissue cDNA to assess gene expression. Primers and probes were obtained from published cDNA sequences by use of the Primer Express program (Applied Biosystems). Amplicons ranged of 50–200 bp were used for optimization, and amplicons crossing an exon junction were chosen to eliminate false-positive results from contaminating genomic DNA. Negative control reactions were also performed, omitting RT, again to ensure lack of contaminating DNA. Positive control reactions were carried out using known amounts of genomic DNA in place of RNA. All primer sets were tested to ensure amplification efficiency. PCRs were done using the ABI Prism 7000 sequence detection system (Applied Biosystems). Oligonucleotide primers used for the analysis were obtained from Integrated DNA Technologies (for CCR5, forward primer of 5'-GCTGAGTCTCCATCTCATCTGATAA-3' and reverse primer of 5'-ATGGCCAGGTTGAGCAGGTA-3'; for CXCR4, forward primer of 5'-TGAGAAGCATGACGGACAGTAC-3' and reverse primer of 3'-GGAAGCGTGATGACAAAGAG-3'). SYBR green was used for CCR5 and CXCR amplex detection. A pre-programmed dissociation protocol was used after amplification to verify that all samples exhibited a single amplicon. Gene expression was normalized to expression of 18S rRNA (Applied Biosystems). Cycling conditions were 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C, and 1 min at 60°C.

**Statistical analyses.** Data that were normally distributed were analyzed using Student’s t test, and data that were not normally distributed were evaluated using the Mann-Whitney U test. \( P \leq .05 \) was considered to indicate statistical significance.

**RESULTS**

**Patient demographics.** Thirty-two women were enrolled in this study. Two subjects were found to be heterozygous for the Δ32-bp deletion in ccr5 and were excluded. There were 2 women with primary syphilis, 4 women with secondary syphilis, 8 women with HSV-1 or HSV-2 infection, 8 women with noninfected vulvovaginal abrasions, and 8 women without lesions (negative control subjects). The mean ± SD age of the women enrolled was 26.1 ± 5.8 years, and the racial distribution in the cohort was as follows: 10 white (37%), 11 African American (37%), and 9 Hispanic (30%). Nine women (64%) with a genital ulcer reported a prior history of a sexually transmitted infection (STI), compared with 6 women (37%) without an ulcer (\( P = .14 \)). Progesterone and estradiol levels did not differ significantly among the groups.

**CCR5 expression on immune cells isolated from genital tissue or peripheral blood.** Immune cells isolated from genital tissue biopsy samples or from peripheral blood were evaluated by 4-color flow cytometry to determine the number of CD3+ and CD14+ cells expressing CCR5. Women with primary syphilis, secondary syphilis, or HSV-1 or HSV-2 infection all had significantly increased numbers of CD14+ cells expressing CCR5 on cell surfaces within tissue obtained from genital ulcers (figure 1). Only patients with primary and secondary syphilis had increased CCR5 surface expression on monocytes isolated from nonulcerated tissue (obtained from the contralateral vulva) (\( P = .006 \) and \( P = .02 \), respectively). In the 4 patients with secondary syphilis, a higher percentage of CD14+ cells isolated from the peripheral blood expressed CCR5, compared with that in the control subjects (mean ± SD, 11.3% ± 2.5% vs. 5% ± 3.1%; \( P = .003 \)). This increase in CCR5+ monocytes in peripheral blood was not observed in the other groups of patients with GUD, consistent with the fact that, during secondary syphilis, T. pallidum is widely disseminated, likely inducing some level of systemic response. There was no increase in CCR5 cell-surface expression on monocytes from women with a nonspecific abrasion. Levels of surface expression of CCR5 on CD3+ cells did not significantly vary among the groups.

To establish whether the increase in CCR5 expression was from selective recruitment of CCR5+ cells alone or also from up-regulation of CCR5 expression on individual cell surfaces, as indicated by increases in the mean fluorescence intensity (MFI) of CCR5, we compared CCR5 receptor density on immune cells isolated from the different groups. Macrophages isolated from primary and secondary syphilis ulcers or from the nonulcerated tissue and peripheral blood of the patients with secondary syphilis had significantly increased levels of CCR5, compared with those isolated from control subjects (figure 2). This increase in MFI was not observed among the macrophages from the HSV-1 or HSV-2 tissue biopsy samples, implying that HSV-1 and HSV-2 infection culminate in the recruitment of CCR5+ cells to the ulcer site rather than specifically inducing the up-regulation of CCR5. No differences in MFI among any of the groups with respect to the CD3+ immune cell population were detected.

**CXCR4 expression on immune cells isolated from genital tissue and peripheral blood.** It has been reported that T. pallidum or bacterial LPS can down-regulate CXCR4 expression on monocytes in an in vitro model and in a human skin blister fluid system [17, 18]. To investigate this further, we evaluated immune cells isolated from genital tissue biopsy samples and from peripheral blood by 4-color flow cytometry to determine the...
number of CD3+ and CD14+ cells expressing CXCR4 (figure 3). A decreased number of monocytes expressing CXCR4 was observed in the peripheral blood of women with primary syphilis, secondary syphilis, or HSV-2 infection ($P = .002, P < .001, \text{ and } P = .02, \text{ respectively}$) but not for women with HSV-1 infection (figure 3). No such decreases were found in the CD3+ peripheral blood immune cells. No altered expression was noted in either of the 2 tissue groups for either CD3+ or CD14+ cells. Interestingly, the MFI of CXCR4 antibody binding was not significantly decreased in either the CD3+ or CD14+ cells isolated from tissue or blood in any of the groups (compared with that in the control subjects) (data not shown).

**qRT-PCR to assess gene expression of CCR5 and CXCR4 in lower genital tract tissue biopsy samples and peripheral blood.** qRT-PCR of RNA isolated from the genital tissues and peripheral blood was performed, and data were compared with the 18S rRNA endogenous control. CCR5 expression was increased 16-fold in tissue obtained from the ulcer site of women with primary syphilis and was increased 32-fold in tissue obtained from secondary syphilis genital lesions ($P < .001$ for each, compared with that in the control samples) (figure 4). This increased expression was not identified in any other tissue group or in the peripheral blood of any of the groups. No alteration of CXCR4 gene expression at the mRNA level was found among any of the groups (data not shown).

**DISCUSSION**

The results of our study provide further insights into potential mechanisms at the cellular level by which GUD acts as a potent facilitator of HIV-1 transmission. These in vivo–derived data show that monocytes recruited to genital ulcer sites express CCR5 in addition to CD4. Our findings also indicate that the amount of this key coreceptor is increased on the cell surfaces, presumably allowing for more efficient viral entry. Increased CCR5 expression on monocytes also was observed among cells in noninfected vulvar tissue and in the peripheral blood of women with syphilis, likely reflecting the systemic nature of syphilis infection. CXCR4, the HIV coreceptor that is not usually involved in heterosexual transmission but, rather, is involved in...
disease progression, was not increased in the setting of GUD and, in fact, was decreased on cells in the peripheral blood of women with syphilis or HSV-2 infection.

GUD ensues on infection with either *T. pallidum*, HSV-1, HSV-2, or *Haemophilus ducreyi*. The epidemiologic relationships between any of these organisms and HIV-1 infection were recognized soon after the onset of the AIDS epidemic [1–8, 23, 24]. Ulcerative STIs are more frequently associated with HIV transmission than nonulcerative STIs, and a recent meta-analysis revealed that GUD increases a woman’s susceptibility to HIV-1 by 3-fold [25–27]. Data from Thailand and Kenya support the finding that genital ulcers increase the per-sex-act risk of HIV infection in countries in which GUD is prevalent [28–30].

GUD as a biological cofactor for HIV-1 transmission is plausible for a number of reasons. HIV-1 and GUD share similar behavioral risk factors and routes of acquisition. Disruption of the protective epithelial barrier by ulceration likely contributes to transmission by facilitating access of the virus to target immune cells. The host immune response to the genital ulcer then recruits HIV-susceptible cells, such as T lymphocytes, dendritic cells, and macrophages [3, 9–13, 31]. It has been proposed by us and others that the immune cells localized to the genital ulcer sites have an increased ability for HIV-1 uptake secondary to up-regulation of the β-chemokine receptor CCR5 [17, 32]. CCR5 is the principal coreceptor for the R5-dependent (M-tropic) strains of HIV. These R5-dependent viruses are the strains predominantly responsible for the majority of the sexually transmitted HIV-1 harbored within macrophages, dendritic cells, and activated or memory T cells [33–38]. T cell–tropic (X4) strains of HIV-1 exploit the α-chemokine receptor CXCR4 and generally infect both naive and established CD4+ T cell lines. These X4 strains are responsible primarily for disease progression and, less frequently, primary transmission.

A few studies have demonstrated that GUD affects HIV-1 coreceptor expression in vitro. *T. pallidum* and its membrane lipoproteins up-regulate the β-chemokine receptor CCR5 on human monocytes in vitro [17]. In an in vivo human skin model, intradermal injection with synthetic *T. pallidum* lipoprotein analogues resulted in enhancement of CCR5 positivity among...
mononuclear cells (compared with that in plasma) [16]. One in vivo study showed that HSV lesions displayed increased CCR5 expression in the setting of increased CD45RO+ T cells and macrophages [19]. Similarly, macrophages isolated from experimentally induced *H. ducreyi* had a significant amount of CCR5 expressed (compared with those in peripheral blood) [32]. Theoretically, differential expression of these coreceptors creates an environment at the site of a genital ulcer conducive to HIV-1 transmission of M-tropic strains. Although biologically plausible, this up-regulation of CCR5 at the site of a genital ulcer has not been demonstrated solidly during naturally occurring GUD. That the female genital tract tissue is accessible experimentally, expresses HIV-1 coreceptors, and is affected by GUD made it ideal for evaluation of HIV-1 coreceptor expression in vivo.

We found that *T. pallidum* infection was associated with significantly increased CCR5 expression on monocytes isolated from genital ulcer tissue. This increase occurred by 2 mechanisms. First, recruitment of CCR5-expressing monocytes to the genital ulcer site was shown by an increase in the percentage of cells expressing this HIV-1 coreceptor. Second, an increase in CCR5 expression on the individual monocytes was shown by both an increase in MFI and an increase in the level of CCR5 mRNA isolated from cells within the syphilitic lesion. In contrast, the increase in CCR5-expressing monocytes found in the nonulcerated tissue on the contralateral vulva and in the peripheral blood from women with secondary syphilis was solely from cell recruitment; no increase in MFI or CCR5 mRNA levels was observed. Women with HSV-1 or HSV-2 infection also had increased numbers of monocytes expressing CCR5, but this increase appears to be secondary to recruitment and did not occur from coreceptor up-regulation.

To establish that nonspecific inflammation did not affect HIV-1 coreceptor expression to the same extent as GUD, women with noninfectious lesions (e.g., trauma from sexual activity) were evaluated. Although the number of inflammatory cells was increased in the biopsy samples of the lesions (compared with that for negative control subjects), HIV-1 coreceptor expression was not significantly increased. Thus, although local trauma may increase inflammation at the site of the lesion, any increase in HIV acquisition in that particular setting likely occurs via the breach in the integument rather than from a facilitation of viral entry by coreceptor up-regulation.

Certain limitations in our study are noteworthy. We were unable to obtain samples from women with chancroid, donovano-

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**Figure 3.** Detection of CXCR4 on CD3+ and CD14+ cells isolated from vulvovaginal tissue and peripheral blood mononuclear cells. Data are expressed as the percentage of cells positive for CXCR4 among the 3 groups (denoted on the X-axis). Data are from the no. of patients times 2 sections from each biopsy or peripheral blood (PB) sample run in duplicate times 2 runs per sample. Results are reported as mean ± SD values from samples studied. Asterisks denote values that differ significantly from the corresponding tissue or PB control values. Gray hatched bars represent CD3+ cells, and black bars represent CD14+ cells.
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Figure 4. CCR5 mRNA in tissue biopsy samples and peripheral blood obtained from women with a genital ulcer or a nonspecific abrasion. Expression was determined by real-time quantitative reverse-transcriptase polymerase chain reaction, and CCR5 gene expression was normalized to the expression of 18S rRNA. Data are presented as relative units relative to 18S. Asterisks reflect \( P < .001 \), compared with CCR5 gene expression in the control women.

sis, or lymphogranuloma venereum. The small size of the genital biopsy sample that could practically be obtained from volunteers also limited the number of cell-surface markers that could be analyzed. Isolating immune cells from a 2.5-mm sample allowed for cells sufficient to perform flow cytometry studies for only 4 cell-surface markers. We chose a panel of markers that provided strategic information relative to our main working hypothesis, but the activation states of the individual cell populations could not be determined, nor could other cell populations be assessed. We also could not determine expression levels of other chemokine receptors such as CCR2b or CCR3, which have been found to have an as-yet poorly understood role in HIV entry.

That GUD is a potent facilitator of HIV-1 transmission is well established. The present study has produced new in vivo–derived data that provide an additional potential mechanism, at the cellular level, to explain this epidemiological relationship. Namely, immune cells recruited to genital ulcer sites express not only CD4, the major receptor for HIV, but also CCR5, a key coreceptor important for efficient viral entry into cells. Given the histopathology of genital ulcers induced by *T. pallidum*, HSV-1, HSV-2, and *H. ducreyi*, it is plausible that all of these STI agents are particularly efficient at promoting HIV coreceptor up-regulation [10, 39–46]. As such, integrated HIV-prevention protocols incorporating behavioral modifications, GUD surveillance and management, and condom use as risk-reduction strategies remain essential for the containment of HIV worldwide. Finally, the HIV-1 coreceptors present in abundance at the site of a genital ulcer potentially provide a pharmacologic target for the development of new interventions to combat HIV acquisition and transmission [47]. Viral entry inhibitors, such as CCR5 antagonists, are currently under development, and the safety and efficacy phase of clinical trials are ongoing [47]. The results presented here provide further rationale for the use of such anti-HIV strategies, particularly in the setting of GUD.

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References
