Cervicovaginal Levels of Lactoferrin, Secretory Leukocyte Protease Inhibitor, and RANTES and the Effects of Coexisting Vaginosis in Human Immunodeficiency Virus (HIV)-Seronegative Women with a High Risk of Heterosexual Acquisition of HIV Infection

Richard M. Novak, 1* Betty A. Donoval, 2 Parrie J. Graham, 1 Lucy A. Boksa, 1 Gregory Spear, 2 Ronald C. Hershow, 1 Hua Yun Chen, 1 and Alan Landay 2

University of Illinois at Chicago 1 and Rush University, 2 Chicago, Illinois

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Innate immune factors in mucosal secretions may influence human immunodeficiency virus type 1 (HIV-1) transmission. This study examined the levels of three such factors, genital tract lactoferrin [Lf], secretory leukocyte protease inhibitor [SLPI], and RANTES, in women at risk for acquiring HIV infection, as well as cofactors that may be associated with their presence. Women at high risk for HIV infection meeting established criteria (n = 62) and low-risk controls (n = 33) underwent cervicovaginal lavage (CVL), and the CVL fluid samples were assayed for Lf and SLPI. Subsets of 26 and 10 samples, respectively, were assayed for RANTES. Coexisting sexually transmitted infections and vaginoses were also assessed, and detailed behavioral information was collected. Lf levels were higher in high-risk (mean, 204 ng/ml) versus low-risk (mean, 160 ng/ml, \( P = 0.007 \)) women, but SLPI levels did not differ, and RANTES levels were higher in only the highest-risk subset. Lf was positively associated only with the presence of leukocytes in the CVL fluid (\( P < 0.0001 \)). SLPI levels were lower in women with bacterial vaginosis [BV] than in those without BV (\( P = 0.04 \)). Treatment of BV reduced RANTES levels (\( P = 0.05 \)). The influence, if any, of these three cofactors on HIV transmission in women cannot be determined from this study. The higher Lf concentrations observed in high-risk women were strongly associated with the presence of leukocytes, suggesting a leukocyte source and consistent with greater genital tract inflammation in the high-risk group. Reduced SLPI levels during BV infection are consistent with an increased risk of HIV infection, which has been associated with BV. However, the increased RANTES levels in a higher-risk subset of high-risk women were reduced after BV treatment.

A number of studies suggest that innate factors in mucosal secretions from a variety of sources are capable of modifying human immunodeficiency virus type 1 (HIV-1) infection in vitro. Secretory leukocyte protease inhibitor [SLPI], a serine protease inhibitor which can be found in oral and genital tract secretions, appeared to inhibit in vitro infection with HIV in several studies (13, 19, 23, 24), although this effect was not consistently found (27). The antiviral effect of SLPI appears to occur in both monocytes and lymphocytes after viral binding to the host cell but before reverse transcription (20), possibly through inhibition of HIV interaction with annexin II (18).

Lactoferrin [Lf], a protein also found in a variety of mucosal secretions, has similarly been shown to have anti-HIV properties (4, 21, 28). RANTES has been demonstrated to be the natural ligand for CCR5 and to be capable of inhibiting HIV infection of macrophages in vitro (2, 5, 7). Both Lf and SLPI have been identified in saliva, and SLPI in an infant’s saliva may play a role in mitigating mother-to-child transmission of HIV (10, 11). SLPI has recently been shown to play a role in the regulation of immunoglobulin class switching at mucosal surfaces (29).

A recent study of commercial sex workers (CSW) who are exposed to HIV but remain uninfected has found elevated RANTES expression in cervical lymphocytes compared with low-risk controls (12) and in HIV-resistant CSW compared to non-HIV-infected CSW (14). There is a paucity of data regarding Lf and SLPI levels in the vaginal mucosal secretions in women with a low or increased risk of acquiring HIV infection that might support a role for these innate antimicrobial factors in vivo, and there has been no study to date which specifically investigated the effect of treatment of BV on genital tract RANTES levels. The present study examined Lf, SLPI, and RANTES levels in cervicovaginal secretions from women with a low or high risk of acquiring HIV infection.

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MATERIALS AND METHODS

Participants and sample collection. Women at high risk for heterosexual acquisition of HIV were recruited as part of a study of behavioral change resulting from participation in a phase 3 preventive vaccine trial (Centers for Disease Control and Prevention Project Vision). Women at low risk were recruited independently. The protocol was approved by the local institutional review board, and written informed consent was obtained in accordance with institutional review board policies for both cohorts. All participants were HIV seronegative and had never injected drugs. High-risk participants (n = 62) had to have had at least one HIV-seropositive sex partner or at least two of the following risk factors: crack cocaine use during the last 6 months; exchange of sex for money, drugs, or shelter during the last 6 months; at least five sex partners.
TABLE 1. Summary of demographics, sexual partners, and practices of study participants

| Parameter                        | Low risk (n = 33) | High risk (n = 62) |
|----------------------------------|-------------------|-------------------|
| Race                             |                   |                   |
| % African-American               | 62.5              | 98.4              |
| % Caucasian                      | 28.1              | 0                 |
| % Asian                          | 9.4               | 0                 |
| % Hispanic                       | 0                 | 1.6               |
| Mean age (yr) ± SD               | 21.8 ± 5.7        | 36.7 ± 6.8        |
| Mean no. of sexual partners ± SD |                   |                   |
| Lifetime                         | 2.7 ± 1.4         | 322.0 ± 435.0     |
| Last 5 yr                        | 2.3 ± 1.1         | 157.9 ± 220.7     |
| Last 6 mo                        | 0.9 ± 0.3         | 58.0 ± 122.5      |
| % With no HIV* partner           | NA*               | 69.8              |
| % With 1 or more HIV* partners   | NA                | 6.4               |
| % Who do not know whether they   | NA                | 23.8              |
| had an HIV* partner              |                   |                   |
| % Condom use*                    |                   |                   |
| Never                            | 12.5              | 20.6              |
| Sometimes                       | 56.3              | 60.3              |
| Always                          | 18.8              | 19.1              |
| NA                              | 12.5              |                   |
| Mean frequency of vaginal sex* ± SD | 24.7 ± 29.4       | 192.3 ± 277.0     |
| No. (%) with BV* ± SD            | 0                 | 15 (24.2)         |

*a During the previous 6 months.

| TABLE 2. Effects of BV and vaginal leukocytes on SLPI and Lf levels |
|---------------------------------------------------------------|
| Factor and subgroup                                           | SLPI concn (pg/ml) | Lf concn (ng/ml) |
| No. of women                                                  |                   |                  |
| BV Absence                                                   | 80                | 9,929 ± 2,862*   |
| Presence                                                     | 15                | 8,865 ± 2,935    |
| White blood cell count*                                       |                   |                  |
| 0                                                            | 10                | 10,278 ± 2,993   |
| 1+                                                           | 19                | 10,096 ± 1,483   |
| 2+                                                           | 25                | 9,567 ± 3,261    |
| 3+                                                           | 41                | 9,533 ± 3,177    |

*The values shown are means and standard deviations. Refer to Table 3 for statistical associations.

** Statistical methods.** Summary statistics were obtained for Lf, SLPI, and risk factors that characterize the low- and high-risk groups (the cohort identity) and other measurements such as erythrocyte and leukocyte counts. The high-risk and low-risk groups were compared for both Lf and SLPI concentrations by the t test. Associations between the response variables (Lf and SLPI) and risk factors and other measurements were examined individually with linear regression models before and after adjusting for the cohort effect. Multivariate linear-regression models were used to examine the association between the response variables (Lf and SLPI, respectively) and the cohort after adjusting for other covariates. The stepwise variable selection procedure was used to find the final multivariate linear models. Analysis of the RANTES data was done by using the random-effect model for repeated measures.

RESULTS

Two cohorts were recruited for this study, 62 high-risk women and 33 low-risk women. Their demographic data have been published elsewhere and can be found in Table 1 (15). Semen was detected in eight samples but was not associated with any of the findings studied, so these samples were included in the analyses (Table 2).

The mean Lf concentration in the high-risk group was 204 ng/ml (standard deviation = 75 ng/ml; range, 19 to 371 ng/ml), which was significantly higher than the mean in the low-risk cohort, which was 160 ng/ml (standard deviation = 70 ng/ml; range, 13 to 252 ng/ml; Fig. 1A) (P = 0.007). The mean SLPI concentration in the high-risk group was 9,987 pg/ml (standard deviation = 3,292 pg/ml; range, 1,832 to 21,469 pg/ml), which did not differ significantly from that of the low-risk cohort, with a mean concentration of 9,257 pg/ml (standard deviation = 70 ng/ml; range, 19 to 371 ng/ml), which was significantly higher than the mean in the low-risk cohort.

The association between the response variables (Lf and SLPI) and risk factors was examined using multivariate linear regression models before and after adjusting for the cohort effect. The associations between the response variables (Lf and SLPI, respectively) and other covariates were also examined individually with linear regression models. The stepwise variable selection procedure was used to find the final multivariate linear models. Analysis of the RANTES data was done by using the random-effect model for repeated measures.
group is represented by women with a range of risk behaviors. We further defined a higher-risk group of women who reported having had $>10$ partners in 6 months and using condoms sometimes or never. When we compared these women to the remainder of the high-risk cohort, they had significantly higher RANTES levels ($P = 0.03$).

The concentrations of SLPI and Lf in the presence or absence of BV or vaginal leukocytes are given in Table 2. It appears that the presence of BV decreases the mean SLPI concentration ($P = 0.04$; Fig. 2B) but increases the mean Lf concentration ($P = 0.004$; Fig. 1B). In addition, increased levels of leukocytes are associated with a decrease in SLPI (statistically insignificant, $P = 0.25$) and an increase in Lf ($P = 0.0002$).

A number of other cofactors that might influence the concentrations of SLPI and Lf were analyzed. With the exception of BV, none of the other risk factors studied was significantly associated with the concentration of SLPI (data not shown). On the other hand, as shown in Table 3, several risk factors, when considered individually, are significantly associated with the concentration of Lf. These include BV, the presence of red...
The presence of leukocytes in the CVL fluid specimen is adjusted for. We collected data on contraceptive and antibiotic use. Only seven participants used hormonal contraceptives. There was no association between type of contraception and either SLPI or Lf. None of the participants reported antibiotic use at the time these samples were taken.

The concentration of SLPI in the CVL fluid samples was not associated with risk behavior. However, the presence of bacterial vaginosis was negatively associated with the SLPI concentration (P = 0.04).

**DISCUSSION**

This study of the innate immune factors SLPI, Lf, and RANTES in the genital secretions of women at high risk for HIV infection but who remain seronegative, compared to a low-risk control group, was able to demonstrate an association of Lf levels with risk behavior, but no similar association existed for SLPI levels with the risk group. RANTES levels were not associated with the risk group, but on further analysis of the high-risk cohort, there did appear to be a positive relationship between RANTES levels and risk behavior. These disparate results for RANTES may reflect a smaller subgroup of the cohort in which this was studied, as well as the use of cervical

![Graph showing RANTES levels](http://cvi.asm.org/)

**FIG. 3.** The mean RANTES levels of the pre- and posttreatment subgroups were 2,428.4 ± 9.307.1 pg/ml and 331.3 ± 632.0 pg/ml (P = 0.05), respectively. Seventeen women had decreased RANTES levels posttreatment, and nine had increased levels.

**TABLE 3. Association between Lf and other factors by linear regression models**

| Variable                          | Univariate analysis slope (P value) | Multivariate analysis regression coefficient (P value) |
|-----------------------------------|------------------------------------|-------------------------------------------------------|
|                                   | Analysis 1                         | Analysis 2                                            | Analysis 1 | Analysis 2 |
| Cohort                            | 44.0 (0.007)                       |                                                       | 49.0 (0.001) |
| Erythrocytes in CVL               | 14.5 (0.03)                        | 13.5 (0.03)                                           |            |            |
| Leukocytes in CVL                 | 27.4 (0.0002)                      | 29.6 (<0.0001)                                        |            |            |
| Semen in CVL                      | 33.9 (0.23)                        | 18.1 (0.52)                                           |            |            |
| STDs                              | 37.7 (0.05)                        | 21.4 (0.29)                                           |            |            |
| Risky sex<sup>b</sup>             | 22.3 (0.18)                        | 0.05 (1.00)                                           |            |            |
| BV                                | 60.8 (0.004)                       | 47.3 (0.03)                                           |            |            |
| Vaginitis<sup>c</sup>             | 62.2 (0.0001)                      | 52.9 (0.004)                                          |            |            |
| Race                              | 31.3 (0.16)                        | 1.9 (0.94)                                            |            |            |
| Age                               | 1.4 (0.09)                         | −0.57 (0.63)                                          |            |            |
| Phase of menstrual cycle<sup>d</sup> | NA (0.91)                         | NA (0.08)                                             |            |            |
| Day in menstrual cycle            | 0.01 (0.31)                        | 0.01 (0.48)                                           |            |            |
| Ever used crack, cocaine, or heroin | 44.4 (0.006)                     | 25.7 (0.50)                                           |            |            |
| Use of crack in past 6 mo         | 44.7 (0.004)                       | 30.3 (0.17)                                           |            |            |
| Crack use frequency in last 6 mo  | 8.0 (0.01)                         | 4.4 (0.27)                                            |            |            |
| Lifetime no. of male sex partners | 0.01 (0.72)                        | −0.02 (0.42)                                          |            |            |
| No. of male partners in past 5 yr | 0.02 (0.67)                        | −0.03 (0.50)                                          |            |            |
| No. of male partners in last 6 mo | 0.01 (0.87)                        | −0.05 (0.55)                                          |            |            |
| Exchanging sex for drugs, money, or shelter | 42.6 (0.007)       | 18.3 (0.68)                                           | 45.9 (0.002) |

<sup>a</sup> Analysis 1, not adjusted for cohort; analysis 2, adjusted for cohort. The variables included in the multivariate analyses were selected by the stepwise selection procedure. A blank entry for a variable in the multivariate analysis results means that the variable was not selected into the model on the basis of an inclusion and exclusion criterion (P < 0.05). NA, not applicable.

<sup>b</sup> Risky sex is defined as having more than five sex partners in 6 months and seldom or never using condoms.

<sup>c</sup> Vaginitis definition includes *Trichomonas* infection and BV.

<sup>d</sup> Phase of cycle was categorized on the basis of days following the last menstrual period as proliferative or luteal. The proliferative phase was defined as the first 15 days after a menstrual period.
sponge samples rather than CVL fluid samples. Additionally, we studied pre- and post-BV treatment samples rather than only baseline samples. These paired samples demonstrated a significant effect of BV treatment on a reduction in RANTES levels. This effect was by no means universal, however, with a third of the women experiencing an increase in RANTES after BV treatment. Other reports of exposed, uninfected women have demonstrated higher levels of RANTES compared to low-risk or HIV-infected women (12, 14). The methods used differed somewhat from those of the present study. Our finding that RANTES levels increased with risk behavior in the high-risk cohort is consistent with these previous reports. Because we chose to study paired samples pre- and posttreatment of BV, potential confounders were better controlled. However, selection bias may exist because the high-risk group does not represent the general population. The effect of BV treatment in the paired samples indicates that BV may raise RANTES levels. Since BV is a highly prevalent infection in high-risk women (24% of this cohort), it may be that the higher RANTES levels seen in other studies may, at least in part, be attributable to coexisting BV or another infection, in addition to the risk group.

None of the specific risk behaviors which define the high-risk group were found to be independently associated with LF. Further analysis of the association with other measured factors after controlling for behavioral risk demonstrates a strong association with the presence of leukocytes, as well as erythrocytes. Since LF is a product of leukocytes, this association is expected and consistent with the high level of vaginal leukocytes we have observed in high-risk women, both with and without a specific diagnosis of vaginosis or vaginitis (16, 26). However, there was also an association of LF with bacterial vaginosis, which was independent of vaginal leukocytosis, unlike Trichomonas infection. Several reports have described the effect of BV on neutrophil numbers and activation and indicated no substantial difference in neutrophil numbers in women with BV compared to healthy controls but a much higher level of inflammatory cytokines in the presence of BV (6). There are numerous studies describing the presence of LF in neutrophilic granules, so it could be inferred that higher levels of neutrophil activation could lead to their degranulation and subsequent release of LF (1, 17).

There have been several reports of BV as a risk factor for HIV transmission in women (22, 25) suggesting that the antiviral effect observed with LF is not adequate to counter the enhancing effect of BV on transmission. The vaginal milieu is complex, and any effect that LF and/or SLPI may have on HIV transmission must be seen in the context of a multifactorial process in which these two factors may be involved.

While no association was seen between SLPI levels and the risk group, there was a significantly lower level of SLPI in women with coexisting BV than in those without BV. In the oral cavity and the genital tract, SLPI, a product of epithelial cells and phagocytes, is degraded by cysteine proteases such as cathespins B, which are increased in inflammatory tissue (8, 9). One role of SLPI is to inactivate elastases, which can damage mucosal surfaces, so it is possible that the inflammation induced by BV may release cysteine proteases which degrade SLPI, as has been demonstrated with Trichomonas infection (9). Alternatively, the organisms or by-products of BV may down-regulate SLPI production or release. If SLPI does have an in vivo effect on HIV infection, this finding is consistent with the enhancing effect of BV on transmission, at least in part, through an effect on the SLPI concentration. Further prospective studies are needed to elucidate the role, if any, of these innate factors in the heterosexual transmission of HIV-1.

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