Mucosal *Escherichia coli* Bactericidal Activity and Immune Mediators Are Associated With HIV-1 Seroconversion in Women Participating in the HPTN 035 Trial

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The mucosal environment may impact the risk for human immunodeficiency virus type 1 (HIV-1) acquisition. Immune mediators were measured in vaginal fluid collected from HPTN 035 participants who acquired HIV-1 and from those who remained HIV-1 negative (controls). Mediator concentrations were similar in samples obtained before as compared to after HIV-1 acquisition in the 8 seroconverters. Compared with controls, seroconverters were more likely to have detectable levels of HβD-2 (odds ratio [OR], 2.39; *P* = .005) and greater *Escherichia coli* bactericidal activity (OR, 1.22; *P* = .01) prior to seroconversion. *E. coli* bactericidal activity remained significant in a multivariable analysis (*P* = .02) and may be a biomarker for HIV-1 acquisition.

Clinical trials evaluating the effectiveness of topical and oral preexposure prophylaxis (PrEP) to prevent human immunodeficiency virus (HIV) acquisition are being conducted in areas of high HIV incidence, notably sub-Saharan Africa, which has the highest incidence in women worldwide [1]. The availability of affordable, female-controlled prevention methods such as topical microbicides would be a major advance to reduce HIV acquisition in this vulnerable group. First-generation topical PrEP products focused on lumenally active drugs designed to maintain the acidic pH of the

female genital tract following coitus (eg, BufferGel) or polysulfated polymers that block HIV from binding to target cells (eg, Carraguard, cellulose sulfate, and PRO 2000). None of these products were effective in large-scale clinical trials [2–5]. Subsequent studies have suggested that biological and behavioral mechanisms contributed to the lack of efficacy, including low antiviral potency, particularly in the presence of semen [6], and to incomplete adherence to product [4]. Other factors, such as concurrent sexually transmitted infections (STIs), hormonal contraception, local inflammation, and loss of protective normal vaginal flora, may confound the results by increasing the risk of HIV infection.

To elucidate the factors that contribute to HIV acquisition, genital tract samples must be collected from study participants and placed into a repository that can be analyzed when the results of the study are available. HPTN 035 studied the safety and effectiveness of BufferGel, PRO 2000, and hydroxyethylcellulose placebo gel, each applied vaginally before coitus, or no gel (condoms only) [2]. A repository of vaginal

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swab specimens was established during the last year of the HPTN 035 trial, which provided the resources to identify potential biomarkers of HIV-1 susceptibility in high-risk populations. In a case-control analysis, we compared vaginal swab eluents from women who acquired HIV type 1 (HIV-1; cases) to women who did not acquire HIV-1 (controls), matched for clinical site and product arm to, determine whether mucosal mediators were associated with HIV-1 acquisition. The mucosal mediators measured included concentrations of cytokines, chemokines, antimicrobial peptides, and bactericidal activity against *E. coli*. The latter may reflect the combined interactions between multiple host immune mediators and contributions from vaginal microbiota [7]. The presence of neutrophils and bacterial vaginosis, by Nugent score, were also assessed.

METHODS

HPTN 035 Cohort and Sampling Procedures

The study was conducted from February 2005 through September 2008 at sites in Philadelphia, Pennsylvania; Blantyre and Lilongwe, Malawi; Durban and Hlabisa, South Africa; Harare and Chitungwiza, Zimbabwe; and Lusaka, Zambia (NCT00074425) [2]. Sexually active, HIV-1-negative women provided consent and were randomized to 1 of 4 arms: Buffer-Gel, PRO 2000, placebo gel, or no gel. Condoms were provided to all participants. The population demographic characteristics, protocol, and trial results are described elsewhere [2]. Gram-stained slides were assessed every 3 months for numbers of neutrophils and Nugent score. Collection of vaginal swab specimens for the repository was initiated at the African sites in 2008. During each quarterly pelvic exam, a Dacron swab was applied to the posterior fornix of the vagina to saturate the tip with fluid and then placed in a cryovial containing 400 μ L of phosphate-buffered saline. The cryovials were stored at -80°C at the sites and shipped to the MTN Network Laboratory after the primary study results were available. Because of Zambian government shipping restrictions, swabs from this site were not included in this analysis.

Vaginal Swab Specimen Processing

Swabs were thawed on wet ice, vortexed for approximately 10 seconds, and compressed against the side of the tube to maximize elution of genital tract secretions. The swab was removed and placed upside down in a new tube and centrifuged at $700 \times g$ for 10 minutes at 4°C . The eluent was removed and placed in the original tube containing phosphate-buffered saline, which was centrifuged to clarify the supernatant. The eluent was divided into 100- μ L aliquots and stored at -80°C until assayed.

Evaluation of Soluble Immune Mediators

Total protein levels were determined using a MicroBCA assay (Thermo Fisher Scientific). Concentrations of interleukin 1 β

(IL-1 β), interleukin 2 (IL-2), interleukin 6 (IL-6), interleukin 7 (IL-7), and macrophage inflammatory proteins (MIPs) 1 α and 1 β were quantified for each sample, using a multiplex proteome array with beads Luminex 100 instrument (Luminex, Austin, TX) and analyzed using StarStation software (Applied Cytometry Systems). Enzyme-linked immunosorbent assay (ELISA) kits were used to quantify levels of lactoferrin (EMD Chemicals, Gibbstown, NJ), SLPI (R&D Systems), human β defensins (H β D) 1–3 (Alpha Diagnostics), and human neutrophil peptides (HNPs) 1–3 (HyCult Biotechnology). Samples above the linear range of the standard curve were diluted using the specific reagent diluent for each ELISA. Bactericidal activity against *E. coli* was measured by mixing bacteria with vaginal eluents or control buffer prior to plating and quantifying colony-forming units (CFUs), as previously described [8]. Results are presented as the percentage reduction in number of CFUs from treated plates relative to control plates.

Statistical Analysis

SPSS version 20.0 (IBM) and Stata version 12.0 (StataCorp) were used for all analyses. Distributions of variables were assessed using histograms. When the percentage of *E. coli* inhibition followed a normal distribution, $\log_{10}$ transformations were necessary to obtain normally distributed data for IL-1 β , IL-6, lactoferrin, SLPI, HNP1-3, and total protein. All other variables (IL-2, IL-7, MIP-1 α , MIP-1 β , H β D-1, H β D-2, and H β D-3) had $\geq 50\%$ of values below the lower limit of detection and were dichotomized at the lower limit. Bacterial vaginosis was defined as a Nugent score of >7 , intermediate vaginal flora was defined as a Nugent score of 4–6, and the white blood cell count (WBC) was dichotomized as minimal neutrophils present (≤ 1) versus several neutrophils present (>1) per microscope field. Paired *t* tests and the McNemar test were used to compare levels of soluble immune mediators before and after seroconversion in HIV-1 seroconverters. Generalized estimating equations with a logit link, robust errors, exchangeable correlation structure, and sampling weights were used to determine the relationship between levels of the soluble immune mediators and the odds of being an HIV-1 seroconverter, using data from the pre-seroconversion visits for women who acquired HIV-1 and their matched controls who did not seroconvert. Spearman correlation coefficients were used to assess the relationship between percentage *E. coli* inhibition and other soluble immune mediators. Similar analyses were used to assess associations with bacterial vaginosis and neutrophils.

RESULTS

The HPTN 035 study enrolled 3101 women, the majority (2901; 93.6%) of whom were from the African sites. A total of 192 women acquired HIV-1 during study participation. Swab specimens were collected from African participants because of

Table 1. Soluble Immune Mediator Levels, Bacterial Vaginosis, and White Blood Count Associated With the Odds of Being a Human Immunodeficiency Virus (HIV) Seroconverter

Variable	OR (95% CI)	P Value	Multivariate Analysis	
			OR (95% CI)	P Value
Log ₁₀ IL-1 β level	1.09 (.68–1.77)	.7		
IL-2 level >LLOD	0.66 (.15–2.98)	.6		
Log ₁₀ IL-6 level	1.27 (.63–2.56)	.5		
IL-7 level >LLOD	1.23 (.38–3.98)	.7		
MIP-1 α level >LLOD	2.52 (.66–9.71)	.2		
MIP-1 β level >LLOD	2.02 (.61–6.72)	.3		
Log ₁₀ lactoferrin level	1.80 (.57–5.67)	.3		
Log ₁₀ SLPI level	0.65 (.24–1.77)	.4		
H β D-1 level >LLOD	0.93 (.25–3.48)	.9		
H β D-2 level >LLOD	2.39 (1.29–4.41)	.005	2.13 (.47–9.54)	.3
H β D-3 level >LLOD	2.87 (.61–13.54)	.2		
Log ₁₀ HNP1–3 level	1.90 (.77–4.67)	.2		
<i>E. coli</i> level, CFUs (% of control) (10% change)	1.22 (1.04–1.43)	.014	1.25 (1.04–1.49)	.02
Total log ₁₀ protein level	25.16 (.51–1229)	.1	15.90 (.32–787)	.2
Bacterial vaginosis	0.70 (.17–2.93)	.6		
Neutrophil level >1 ^a	2.64 (.67–10.48)	.2		

Generalized estimating equations with a logit link, robust errors, exchangeable correlation structure, and sampling weights were used to determine the relationship between levels of the soluble immune mediators and the odds of being an HIV-1 seroconverter, using data from the preseroconversion visits for women who acquired HIV-1 and their matched controls who did not seroconvert.

Abbreviations: CFUs, colony-forming units; CI, confidence interval; *E. coli*, *Escherichia coli*; H β D, human β defensin; IL-1 β , interleukin 1 β ; IL-2, interleukin 2; IL-6, interleukin 6; IL-7, interleukin 7; LLOD, lower limit of detection; MIP, macrophage inflammatory protein; OR, odds ratio.

^a White blood cell count was dichotomized as minimal neutrophils present (≤ 1) versus several neutrophils present (> 1) per microscope field.

the higher HIV-1 infection incidence at these sites. In the final year of the study, 3524 vaginal swab specimens were collected from 2031 women. Among women who acquired HIV-1, 26 swab specimen were collected within 3 months of seroconversion from 8 seroconverters; 11 swab specimens were collected prior to seroconversion. We compared swab specimens obtained before and after seroconversion to determine whether HIV-1 acquisition was associated with changes in soluble mucosal immune factors. No significant differences were observed in any of the mucosal measures (data not shown).

Each seroconverter was matched to 3 controls for product, site, and number of available samples, and preseroconversion eluents ($n = 9$ swab specimens from 8 women) were compared to control eluents ($n = 47$ swab specimens from 24 women) (Table 1). Women who seroconverted to HIV-1 were more likely to have detectable levels of H β D-2 (odds ratio [OR], 2.39; $P = .005$) and greater *E. coli* bactericidal activity (OR, 1.22; $P = .01$). There was also a trend toward higher concentrations of protein in samples from women who seroconverted, compared with the controls. In a multivariable model of factors associated with HIV-1 acquisition, which included total protein level, H β D-2, and anti-*E. coli* activity, only anti-*E. coli* activity remained significant ($P = .02$) (Table 1). Results

were similar when controlling for herpes simplex virus 2 infection status.

DISCUSSION

Changes in mucosal immunity may contribute to HIV-1 susceptibility and to the efficacy of PrEP strategies. Epithelial cells lining the mucosa and resident or recruited immune cells are responsive to environmental stimuli, resulting in changes in mucosal concentrations of cytokines, chemokines, and antimicrobial peptides, referred to as the soluble innate immune response [9]. We used vaginal swab specimens from a repository that were collected during a multicenter microbicide study to explore this concept. No significant differences in mucosal immune mediators before and after seroconversion were detected, which is consistent with a study from South Africa, which found that cytokine concentrations before seroconversion did not differ from those after seroconversion ($n = 22$) [10]. However, women from HPTN 035 who seroconverted to HIV-1 had greater *E. coli* bactericidal activity in their preseroconversion vaginal fluid, compared with control women who did not seroconvert. These changes may reflect subclinical

responses to environmental stimuli that could increase their susceptibility to HIV-1.

Our results also suggest that increased *E. coli* bactericidal activity may provide a novel biomarker for increased HIV-1 susceptibility. The factors that contribute to and regulate the bactericidal activity of genital tract secretions and their biological significance are not well-defined, but we hypothesize that this functional assay may provide a more comprehensive measure of mucosal immunity, compared with measures of individual selected mediators [7]. Notably, the bactericidal activity did not correlate with concentrations of individual immune mediators (data not shown). Ongoing studies suggest that the activity may reflect contributions from proteins and other molecules secreted by genital tract epithelial and immune cells, as well as by microbiota [7, 8].

Potential stimuli that may predispose women to HIV-1 acquisition and are possibly linked to the elevated bactericidal activity, higher levels of mucosal immune mediators, and increased numbers of neutrophils include concurrent STI, changes in vaginal microbiota, use of hormonal contraception, and sexual practices. The majority of participants were using depot medroxyprogesterone acetate, and no differences between cases and controls were found. All participants were screened for common STIs and were evaluated monthly for genitourinary signs and symptoms, although this does not preclude intermittent silent infections, such as those involving herpes simplex virus or human papillomavirus [2]. While bacterial vaginosis was relatively common during follow-up, no association was found between its detection and *E. coli* bactericidal activity or increased HIV-1 acquisition. These findings differ from those of a small US study in which bacterial vaginosis was associated with a reduction in *E. coli* bactericidal activity and was partially restored following treatment with metronidazole [11]. These discrepancies may reflect differences in vaginal microbiota or other factors between US and African populations.

It is possible that genetic polymorphisms in defensins, antimicrobial peptides, and other molecules that comprise differences in mucosal innate immunity contribute to the risk for HIV-1 acquisition. Polymorphisms have been identified in persons with Crohn's disease that reduce epithelial permeability [12], and single-nucleotide polymorphisms in pattern recognition receptors that recognize mucosal pathogens are associated with mucosal immune dysregulation resulting in suboptimal pathogen clearance [13] and induction of proinflammatory cytokines [14]. While these studies have focused on inflammatory bowel disease, similar polymorphisms could impact the homeostasis of the female genital tract [15]. Whether similar polymorphisms contribute to our findings will require larger, longitudinal studies that incorporate some of the candidates identified here (eg, *E. coli* bactericidal activity), concentrations of inflammatory mediators, as well as

genetic and epigenomic testing to identify biomarkers associated with increased HIV-1 susceptibility.

There are several limitations to the current study. Collection of vaginal swab specimens was added to the HPTN 035 protocol and initiated relatively late into the trial. Thus, samples obtained before and after seroconversion were available from only 8 subjects. Second, while vaginal swab specimens are easier to collect, endocervical swab specimens may provide a better representation of mucosal immunity at the site of HIV-1 infection. Higher levels of many mucosal mediators were detected by endocervical swabs or cervicovaginal lavage, compared with vaginal swabs, presumably reflecting the greater numbers of immune cells in the cervix as compared to the vagina [8].

Despite these limitations, our results support the contention that the mucosal environment plays an important role in protecting against or facilitating HIV-1 infection. The findings are similar to results obtained in South Africa from CAPRISA 002, a longitudinal prospective study of women at risk for HIV-1, and from CAPRISA 004, a study of tenofovir gel [10]. Moreover, these findings suggest that a functional bioassay measuring bactericidal activity against *E. coli* may provide a biomarker of HIV-1 susceptibility.

Notes

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