Published in final edited form as:
AIDS. 2010 August 24; 24(13): 2069–2074. doi:10.1097/QAD.0b013e32833c323b.

Increased Levels of Immune Activation in the Genital Tract of Healthy Young Women from sub-Saharan Africa

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Abstract

Objectives—To determine if healthy, young women in sub-Saharan Africa have a more activated immune milieu in the genital tract (i.e. activated CD4+ T-cells) than a similar population in the US.

Design—Cross-sectional study nested in a phase 1 microbicide trial.

Methods—Cervical cytobrushes were collected from 18–24 year old women in San Francisco, CA (n=18) and Kisumu, Kenya (n=36) at enrollment into a phase 1 microbicide trial. All participants tested negative for HIV, HSV-2, gonorrhea, chlamydia and trichomonas, and had abstained from sex for at least seven days prior to enrollment. Cryopreserved T-cell populations were assayed by flow cytometry in a central laboratory. SLPI levels were assayed in cervicovaginal lavage samples. The Wilcoxon rank-sum test was used to compare immune parameters between sites.

Results—The total number of endocervical CD4+ T-cells was slightly higher in San Francisco, but participants from Kisumu had a substantially higher number and proportion of CD4+ T-cells expressing the early activation marker CD69, with and without the HIV-coreceptor CCR5, and a greater proportion of activated CD8+ T-cells. Median [interquartile] genital levels of SLPI were lower in participants from Kisumu compared to those from San Francisco (190 pg/mL [96, 519] vs. 474 pg/mL [206, 817]; $p<0.03$).

Conclusions—Activated mucosal T-cells were increased in the genital tract of young, STI/HIV-free Kenyan women, independent of common genital co-infections, and SLPI levels were reduced. The cause of these mucosal immune differences is not known, but could partly explain the high HIV incidence in young women from sub-Saharan Africa.

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Keywords

HIV transmission; mucosal immunology; female genital tract; sub-Saharan Africa; CD4+ T-cells; SLPI

Introduction

Women in sub-Saharan Africa have a higher HIV prevalence than men, and a 80–300 times higher HIV-1 (HIV) incidence than women in the US [1–3]. These geographic and gender differences may be multifactorial [4,5], but it is known that mucosal immune activation resulting from genital tract infection or the application of nonoxynol–9 increases mucosal expression of the HIV coreceptor CCR5 [6], the number of activated CD4+ T-cells [7,8] and the levels of pro-inflammatory cytokines [9,10].

We hypothesized that regional differences in HIV acquisition by young women could be partly due to differences in the genital tract immune milieu, particularly since increased systemic immune activation has been seen in individuals from sub-Saharan Africa [11]. We examined this question in the context of a phase 1 microbicide safety trial with sites in the Kenya and the US, in which all participants had undergone screening for a wide array of genital co-infections. Specifically, we hypothesized that young Kenyan women without genital infections would have increased mucosal activated CD4+ T-cells and pro-inflammatory cytokines relative to a similar US population, leading to greater HIV susceptibility.

Methods

Design

We conducted a cross-sectional study of women at enrollment into a phase 1, placebo-controlled, randomized, double blind clinical trial in sexually-abstinent young women in San Francisco, USA, and Kisumu, Kenya. This trial is registered at www.ClinicalTrials.gov (NCT00331032).

Selection of subjects

Participants provided informed consent, and all women enrolled in the trial were included in this substudy. Eligible participants were 18–24 years old, in good health, sexually experienced, sexually abstinent one week prior to enrollment, not breastfeeding or pregnant and not within 3 months of last pregnancy, and had regular menstrual cycles of at least 25 days. At screening, women were tested for genital infections and pregnancy. Women who had any of the following were excluded: positive test for human chorionic gonadotropin (hCG), urinary tract infection, HIV antibodies, HSV-2 antibodies, syphilis, vaginal candidiasis, symptomatic bacterial vaginosis (BV), a Nugent score ≥7 [21], trichomonas, gonorrhea, chlamydia, abnormal cervical cytology, >2 vaginal infections in the past year, an uncontrolled medical condition or recent acute illness; a recent new systemic or topical medication, or any vaginal product 30 days prior to enrollment. All participants were enrolled between the 5^{th} and 14^{th} day of the menstrual cycle. A pelvic examination including colposcopy, vaginal pH, Gram stain and vaginal wet mount were performed. Vaginal specimens were collected for prostate specific antigen (PSA) testing [12]. A cervical cytobrush was collected and placed into 5ml of cellular transport medium (RPMI with 10% FBS), stored at 4°C and transported to the laboratory on ice. A cervicovaginal lavage (CVL) was performed with 5ml of phosphate buffered saline (PBS), reaspirated and

transported to the laboratory on ice. All samples used were collected prior to administration of the vaginal microbicide.

Lab methods

Cervical samples were tested for 37 HPV genotypes and Beta-globin (Roche Molecular Diagnostics, Inc., Alameda, CA) [13]. Cervical cells from cryopreserved specimens stored at −150°C were stained in two aliquots [14]; one with CD69-FITC, CCR5-PE, CD4-PerCP and CD3-APC (BD Pharmingen, San Jose, California, USA); the other with CD1a (Imgenex San Diego, California, USA), CD11c, CD14 and DC-SIGN (eBioscience, San Diego, California, USA). Samples were acquired by FACSCalibur (Becton-Dickinson Immunocytometry Systems). Cell numbers were multiplied by two to determine 'cells per cytobrush' and $\log_{10}$ transformed for analysis.

Cytokines were assayed in thawed CVL using the LINCOplex High-Sensitivity Human Cytokine Immunoassay Kit (Millipore, Billerica, MA) and the Luminex-100 platform (Luminex, Austin, TX). Secretory leukocyte protease inhibitor (SLPI) was measured by ELISA (Quantikine Human SLPI kit, R&D Systems).

Statistical Analysis

Site-specific distributions of cell counts, SLPI, and cytokine values were summarized via median and interquartile values. Wilcoxon rank-sum tests were used to test factors by site. The proportions of immune cells were defined as the percent of a specific cell phenotype (e.g. CD4 T-cells) that expressed a cell surface ligand(s) (e.g. CD69). Proportions were compared between sites using negative binomial regression. Predictor variables for regression models were selected based on biological plausibility for associations with immune outcomes and observed associations with study site. Variables significantly associated with study site at the 10% significance level were considered as candidates for adjustment. Linear regression models were used to investigate the possible confounding influence of these variables.

Results

Enrollment and participant disposition

A total of 54 participants were enrolled, 18 from San Francisco and 36 from Kisumu (Table 1). Women in Kisumu were younger, less likely to use oral contraceptives, had fewer lifetime number of sex partners, and were less likely to report anal sex and a reproductive tract infection. No differences were seen for recent number of sex partners. No participants had BV at screening, but by the enrollment visit (up to 30 days later) 5 (14%) and 4 (11%) participants from Kisumu had asymptomatic BV or intermediate flora, respectively, compared to no participant from San Francisco (Table 1). HPV was detected in 11 (33%) and six (35%) of women in Kisumu and San Francisco, respectively. No participant had PSA detected (≥1 ng/ml), indicating a lack of recent (within 2 days) semen exposure [12].

Flow cytometry results

The total number of cervical CD4+ T-lymphocytes was slightly higher in women from San Francisco than from Kisumu (304 vs. 187 per cytobrush; $p=0.05$). However, the number and proportion of CD4+ cells expressing CD69+, with or without CCR5+, was substantially higher among women from Kisumu (both $p<0.0001$; Table 2). Similarly, while the number of CD8+ T-lymphocytes tended to be greater in San Francisco women, the proportion of cervical CD8+ T-cells expressing CD69+ was higher in Kisumu (Table 2). The number of CD1a+, CD11c+ and CD14+ cells, but not the proportions expressing DC-SIGN+, were greater in San Francisco (Table 2).

In multivariate analysis, controlling for age, age at first sex, and lifetime and past 3-month number of sex partners did not significantly affect the association between site and flow cytometry results (data not shown). Since HPV infection could putatively influence genital immunology [15], we controlled for HPV status in multivariate analysis. The number and proportion of CD4+/CD69+ ($p<0.007$, $p=0.003$, respectively) and CD4+/CD69+/CCR5+ ($p=0.02$, $p<0.002$, respectively), and proportion of CD8+/CD69+ ($p<0.003$) remained statistically greater in Kisumu compared to San Francisco participants.

Current use of combined oral contraceptives was not associated with differences in cervical cell populations in San Francisco participants, the only site where participants reported use of combined oral contraceptives (data not shown). In Kisumu, altered vaginal flora (Nugent score 4–6) was associated with decreased cervical CD1a+ ($p=0.01$) and CD1a+/DC-SIGN+ ($p=0.02$) cell numbers, but not with other differences. Neither the current use of combined oral contraceptives (San Francisco site only) nor abnormal vaginal flora (Kisumu site only) significantly affected the overall study findings of greater T-cell activation in the cervix of young women from the Kisumu site.

Cervicovaginal levels of SLPI and cytokines

The median CVL concentration of SLPI was significantly lower in participants from Kisumu than from San Francisco (190 vs. 474 pg/ml; $p=0.009$; Table 2); SLPI concentration was not associated with abnormal vagina flora (170 vs. 196 pg/ml; $p=0.83$) at the Kisumu site. No consistent differences were seen in cytokine/chemokine levels between the two sites, although levels of IL-2 ($p<0.02$) levels were higher in Kisumu participants, and IL-8 ($p<0.04$) and GM-CSF ($p<0.004$) were higher in San Francisco (Table 2). At the San Francisco and Kisumu sites GMCSF was bellow the limit of detection (BLD) in 5 (28%) and 23 (64%) cases and IL-2 was BLD in 12 (67%) and 14 (39%) cases, respectively. When these were dichotomized as “detectable” or “not detectable”, both IL-2 ($p<0.05$) and GM-CSF ($p<0.01$) remained associated with site.

Discussion

In this study, young healthy women from Kenya had a higher number and proportion of activated endocervical CD4+ T-cells than women from the US. Since a critical mass of activated CD4+ T-cells at the site of HIV exposure may be essential for local HIV propagation and subsequent systemic dissemination after sexual exposure [19], this might explain in part the increased risk of HIV acquisition in women from sub-Saharan Africa [1–3]. While it has been suggested that reasons for the discrepancy in HIV-seroprevalence include higher prevalence of STIs, specifically HSV-2 [16,17], as well as structural and sociocultural factors [4,5], for the first time our observations suggest that differences in the genital tract immune milieu may be an important additional contributor. The cellular findings were somewhat surprising since we also observed a ~2-fold increased concentration of IL-8, a pro-inflammatory chemokine [18], in the CVL of participants in San Francisco, suggesting that these observations were independent. In addition, we found that the young women from Kisumu had lower cervicovaginal levels of SLPI. SLPI, an innate protein, has been shown to protect activated CD4+ T-cells from HIV infection *in vitro* [20], and is reduced in bacterial vaginosis [21] and following application of nonoxynol–9 [10].

The mechanism underpinning this increase in activated genital CD4+ T-cells will be an important topic for future investigations. Prior studies have shown increased systemic immune activation in several African populations [11], but to our knowledge this is the first to compare the genital immune milieu between African and US participants. Although we controlled for confounders such as genital co-infections, sexual behavior, menstrual phase and hormonal contraception, it may be that genital immune activation stemmed from

differences in host genetics, or from chronic infections that are systemic (e.g. malaria) or affect other mucosal sites (e.g. helminths, gastroenteritis, or schistosomiasis) and are more prevalent in sub-Saharan Africa [22]. In theory, infections at non-genital mucosae such as the gut may not only recruit activated, antigen-specific CD4+ T-cells to that site, but also to other mucosal surfaces such as the genital tract, perhaps through expression of the mucosal homing receptor integrin α4β7 that is expressed at both sites [23,24]. The cause of reduced SLPI concentrations is less clear, although disturbances in the genital tract flora may affect its concentration [21].

Although all flow cytometry was conducted at a single laboratory, it is important to consider whether sampling differences between sites might have contributed to the observed differences, particularly given that the overall numbers of most cervical cell types were actually higher at the San Francisco site. However, standard operating procedures were used to collect, cryopreserve and ship the specimens to the central laboratory, and training at the clinical sites was performed by the same investigator.

While other factors such as physical, behavioral and structural conditions certainly put young women in sub-Saharan African at greater risk of HIV acquisition, ours is the first study to demonstrate that mucosal immune differences in the genital tract may also be important. In addition to stimulating further research, we believe that these findings should also start to dispel some of the preconceptions and stigma surrounding HIV acquisition among young women in sub-Saharan Africa.

Acknowledgments

This study was supported by the STI Clinical Trials Group (NIAID-DMID HHSN266200400074C). In addition, this study was supported by NIH/NCRR UCSF-CTSI Grant Number UL1 RR024131 and the Canada Research Chair Program (R.K., salary support). Its contents are solely the responsibility of the authors and do not necessarily represent the official view of the NIH. Information on NCRR is available at http://www.ncrr.nih.gov/. Information on Re-engineering the Clinical Research Enterprise can be obtained from http://nihroadmap.nih.gov/clinicalresearch/overview-translational.asp.

We would like to thank Jonathan Glock, Carolyn Deal, Shacondra Brown, the VivaGel study teams in San Francisco and in Kisumu, Sanja Huibner for technical assistance, the collaborating laboratories, the STI Clinical Trials Group, National Institutes of Allergy and Infectious Diseases Division of Microbiology and Infectious Diseases, the study participants for volunteering their time and information, and the Director KEMRI.

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Table 1

Comparison of sociodemographic factors and sexual history between women, aged 18–24 years, enrolled in Kisumu, Kenya and San Francisco, USA

	Kisumu N = 36	San Francisco N = 18	*p*-value*
Age (mean ± SD)	20.4 ±1.6	21.7 ± 1.9	0.02
Years of formal education (mean ± SD)	13.2 ± 2.2	14.9 ± 1.4	0.001
Current marital status			
Married	0	2 (11%)	-
Single	36 (100%)	16 (89%)	0.10
Race/Ethnicity			
Asian	0	3 (17%)	-
Black or African American	0	1 (6%)	-
White	0	13 (72%)	-
Kenyan	36 (100%)	0	-
Unknown	0	1 (6%)	<0.0001
Pregnancy History			
Number of pregnancies in lifetime (mean ± SD)	0.2 ± 0.4	0.2 ± 0.4	0.81
Vaginal birth	6 (17%)	1 (6%)	0.40
Miscarriage or termination of pregnancy	0	2 (11%)	0.11
Contraceptive Use			
Current use condoms	17 (47%)	7 (39%)	0.56
Current use combined oral contraceptives	0	7 (39%)	<0.0001
Current use injectable/subdermal hormones	3 (9%)	2 (11%)	1.0
Sexual History			
Age at first sex (mean ± SD)	17.6 ± 2.5	16.7 ± 1.6	0.12
Regular sexual partner	25 (70%)	14 (78%)	0.75
Number of sexual partners in lifetime (mean ± SD)	1.7 ± 0.8	6.0 ± 6.2	0.01
Number of male sexual partners in last 3 months (mean ± SD)	0.8 ± 0.5	1.0 ± 0.7	0.18
Number of episodes of vaginal sex in past month (mean ± SD)	0.9 ± 1.2	6.3 ± 10.5	0.04
STI History			
Ever had reproductive tract infection	3 (8%)	11 (61%)	<0.0001
Lifetime number of STI diagnoses (mean ± SD)	0.1 ± 0.3	0.2 ± 0.4	0.37
Vaginal Gram stain results			
Normal flora (score 0 – 3)	27 (75%)	18 (100%)	-
Intermediate flora (score 4 – 6)	4 (11%)	0	-
Bacterial vaginosis (score 7 – 10)	5 (14%)	0	0.09
Human Papilloma Virus	11 (33%)	6 (35%)	0.89

* *P*-values for comparisons of continuous variables are based on a Student's T-test while *p*-values for comparisons of categorical factors are based on Fisher's Exact or χ^2 test.

Table 2

Comparison of cervical immune cell populations and soluble immune factors in cervicovaginal lavage in 18–24 year old women from Kisumu, Kenya and San Francisco, USA.

Variable	Kisumu		San Francisco			
	Median [IQR]	Proportion [IQR]	Median [IQR]	Proportion [IQR]	*p*-Value*	*p*-Value†
CD4+	187 [31, 403]	-	304 [200, 640]	-	0.05	-
CD4+/CD69+	51.5 [11.5, 91.9]	15.7 [8.3, 56.7]	10 [6, 23]	3.6 [2.3, 6.5]	0.006	<0.00001
CD4+/CCR5+	119 [24, 253]	66.5 [57, 84.2]	205 [95, 321]	60.2 [43, 78]	0.15	0.23
CD4+/CD69+/CCR5+	43 [9, 94]	15.3 [6.7, 45.0]	9 [5, 21]	2.7 [1.9, 6.2]	0.01	<0.002
CD8+	143 [38, 305]	-	230 [142, 526]	-	0.08	-
CD8+/CD69+	36 [8, 86.5]	25.8 [8.7, 59.6]	12 [6, 35]	5.1 [3.5, 9.6]	0.11	<0.0001
CD1a+	134 [90, 330]	-	320 [215, 616.5]	-	0.03	-
CD1a+/DC-SIGN+	90 [69, 123]	66.7 [45.3, 84.6]	197 [79, 361]	65.4 [44.8, 82.4]	0.038	0.90
CD11c+	267 [130, 791]	-	1285 [279, 2705]	-	0.01	-
CD11c+/DC-SIGN+	113 [80, 213]	56.7 [31.5, 71.7]	301 [118, 831]	44.5 [15, 57.4]	0.01	0.31
CD14+	356 [121, 473]	-	662 [249, 1067]	-	0.05	-
CD14+/DC-SIGN+	167 [74, 342]	79.2 [62.3, 86.1]	221 [162, 647]	62.1 [30.1, 82.1]	0.20	0.85
Concentration pg/mL						
SLPI	190 [96, 519]	-	474 [206, 817]	-	0.009	
GM-CSF	0.06 [0.06, 0.3]	-	0.6 [0.06, 1.3]	-	0.004	
IFN-γ	0.15 [0.06, 0.7]	-	0.5 [0.2, 0.8]	-	0.15	
IL-2	0.2 [0.06, 0.7]	-	0.06 [0.06, 0.2]	-	0.02	
IL-4	0.3 [0.06, 0.8]	-	1.7 [0.06, 6.8]	-	0.06	
IL-6	10.8 [3.3, 33.9]	-	17.3 [5.7, 27.7]	-	0.5	
IL-8	166 [85.5, 444]	-	345 [146, 1,500]	-	0.04	
IL-10	0.4 [0.1, 2.1]	-	0.3 [0.06, 307.1]	-	0.59	
IL-12	0.1 [0.06, 0.2]	-	0.1 [0.06, 0.2]	-	0.32	
IL-13	0.06 [0.06, 0.4]	-	0.08 [0.06, 0.4]	-	0.54	
IL-1β	0.2 [0.06, 1.1]	-	1.0 [0.06, 12.0]	-	0.08	
TNF	0.1 [0.06, 0.7]	-	0.06 [0.06, 0.4]	-	0.75	

* *p*-Value represents comparison of number of cells between sites using the Wilcoxon Rank-Sum Test

† *p*-Value represents comparison of proportion of cells between sites using negative binomial regression