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Changes in genital tract immune cell populations after initiation of intrauterine contraception

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OBJECTIVE: The primary target cells for the human immunodeficiency virus (HIV) infection in the genital tract are CD4 T cells that express CCR5 on the surface. Alterations in genital tract T cells that express CCR5 could impact HIV acquisition risk. We hypothesized that, when compared with baseline, the use of a hormonal intrauterine device (IUD) would alter HIV target cells (primarily CCR5+CD4 cells) in the female genital tract more than a nonhormonal IUD.

STUDY DESIGN: Thirty-four healthy HIV-negative women aged 18-40 years who were seeking an IUD for contraception were assigned randomly to receive a levonorgestrel IUD or a copper T380A IUD. A parallel group of 8 control women who did not need contraception was also enrolled. Genital tract mucosal immune cell populations that were collected by cervical cytobrush and endometrial biopsy before and 2 months after IUD placement were analyzed by flow cytometry. Mean differences in cell number and percent that expressed receptors from baseline to follow-up examination were evaluated with the use of paired Student t tests.

RESULTS: Neither IUD altered the number of T cells within the upper and lower genital tracts. Levonorgestrel IUD users had a decrease in T cells that expressed the HIV coreceptor CCR5 in the endometrium and cervix after 2 months of use compared with baseline. There was a decrease in activated endometrial T cells in levonorgestrel IUD users and a decrease in activated cervical T cells in copper IUD users after 2 months of IUD use, compared with baseline.

CONCLUSION: Women who use IUDs have reduced expression of the CCR5 HIV coreceptor on T cells in the endometrium and cervix compared with expression before IUD placement. These findings suggest that susceptibility to HIV infection would not be increased by IUD use.

Key words: CCR5, HIV, hormonal contraception, IUD, T cell


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HIV acquisition and sexual transmission are dependent on the immune environment of the female genital tract and may be under hormonal regulation. Endogenous sex hormones vary through the menstrual cycle, and exogenous hormonal exposure occurs commonly with contraceptive use, which may influence mucosal immune cellular populations.

Mucosal CD4 T lymphocytes in the vagina and cervix are thought to be the primary targets for sexual transmission of HIV to women. However, observed increased risk of HIV acquisition during pregnancy. However, progesterone may contribute to the expression in the setting of increased progesterone. In vivo, increased CCR5 expression is enhanced by sex hormones as ex vivo studies have demonstrated stimulation of CCR5 within explanted cervical tissue when incubated in media containing progesterone. Antigen-presenting cells, such as mucosal dendritic cells, monocytes and macrophages (CD14+ cells) may help transport HIV from the surface to underlying target cells.

CCR5 is expressed on genital tract T cells and is the predominant target coreceptor for initial HIV infection. CCR5 expression is enhanced by sex hormones as ex vivo studies have demonstrated stimulation of CCR5 within explanted cervical tissue when incubated in media containing progesterone. In vivo, increased CCR5 expression in the setting of increased progesterone may contribute to the observed increased risk of HIV acquisition during pregnancy. However, there are limited data on lymphocyte changes with use of COCs and DMPA; there are no data on genital tract immune cell populations within reproductive tract mucosa of women who use other contraceptive methods, including IUDs.

We hypothesized that genital-tract immune cell populations, particularly CCR5+ T cells, would be increased from baseline 2 months after the initiation of intrauterine contraception with a levonorgestrel IUD (LNG-IUD) that contained 52 mg of LNG more than after the initiation of use of a nonhormonal copper IUD (Cu-IUD). We hypothesized that concentrated progestin exposure in the genital tract would recruit HIV target cells to the area. We tested this hypothesis by examining immune cellular populations in upper and lower genital tract samples that were obtained immediately before and 2 months after IUD insertion in healthy women who were assigned randomly to receive an LNG-IUD or Cu-IUD. Because IUDs are placed directly into the uterus, we hypothesized that the greatest impact on genital lymphocytes would be observed in T cells that were recovered from endometrial biopsy specimens.

Materials and Methods

We performed a randomized study of women who were initiating intrauterine contraception plus a parallel control group of women who were not at risk of pregnancy because of heterosexual abstinence or previous surgical sterilization. The primary objective was to assess the impact of IUD initiation on T cells in the upper and lower genital tract. The University of Pittsburgh Institutional Review Board approved this study. All participants were enrolled at the Center for Family Planning Research, Magee-Womens Hospital of the University of Pittsburgh Medical Center and signed informed consent before study participation.

Forty-two women, 18-40 years old, were enrolled; 34 women were seeking an IUD for contraception, and 8 women who were not seeking contraception comprised an observational control group. Eligible women were healthy, HIV negative, and nonpregnant and had regular menstrual cycles. All enrolled study participants were free of genital tract infection on screening examination, including rapid testing for Trichomonas vaginalis (OSOM; Sekisui Diagnostics, Lexington, MA), yeast vaginitis, symptomatic bacterial vaginosis by Amsel's criteria, and abnormal inflammation (>10 white blood cells/high-power field on wet mount). Women were excluded if within 60 days of enrollment they used any hormonal or intrauterine contraceptive, were pregnant or breastfeeding, underwent any genital tract procedure (including biopsy), were diagnosed with any genital tract infection, and/or had a new sexual partner. Exclusion criteria included the use of DMPA within 10 months of enrollment; the use of oral or vaginal antibiotics, oral or vaginal steroids, or any vaginal product except tampons (such as spermicide, microbicide, douche, antifungal, steroid, or hormone) within 30 days of enrollment; a contra-indication to IUD use or an allergy to any component of the IUDs; or a previous malignancy of the cervix or uterus. Women in the control group had to be not at risk of pregnancy, which was defined as heterosexually abstinence or surgically sterile.

Screening also included urine pregnancy testing, collection of blood to rule out HIV infection, and collection of cervical swabs for detection of Neisseria gonorrhoeae and Chlamydia trachomatis by nucleic acid amplification testing (Gen-Probe, San Diego, CA). One participant was found to be ineligible after enrollment because of chlamydial infection, and a second participant withdrew from the study after IUD insertion; both were replaced to maintain the targeted sample size.

Participants were enrolled immediately after screening if eligible that day or returned for enrollment on a day when no vaginal bleeding was present. Day of menses at the time of enrollment was recorded. Participants were asked to refrain from any vaginal or anal intercourse for 1 week before sample collection at both visits. The 34 women who were seeking an IUD for contraception were assigned randomly 1:1 to receive either an LNG-IUD (Mirena; Bayer HealthCare Pharmaceuticals, Wayne, NJ) or copperT380A IUD (ParaGard; Teva Pharmaceuticals, Sellersville, PA). At the time of random assignment, the study investigator opened the next sequentially numbered, opaque, sealed envelope that contained the group assignment of LNG-IUD or Cu-IUD. A statistician who was not involved with the clinical conduct of the study prepared the envelopes using computer-generated random allocations in permuted blocks. The IUD was inserted per standard clinical practice at the enrollment visit immediately after the collection of all study samples. All laboratory personnel were masked to clinical status of participants, including random assignment to IUD type.
A, Live single cells were identified from the lymphocyte population. CD4 and CD8 T cells were identified from CD45CD3$^+$ populations; CCR5 and CD69 positive cells were identified from both CD4 and CD8 cells. B, Single cells were identified from the neutrophil, macrophage, dendritic cell populations; live CD14 cells were gated from this population of single cells.

Genital tract samples were collected at enrollment and at 8-week follow-up visits. Endocervical specimens were obtained with a cytobrush (CooperSurgical Inc, Trumbull, CT) that was inserted into the cervical os and rotated 360 degrees; the cytobrush was placed in 4-mL Roswell Park Memorial Institute (RPMI) medium (Mediatech Inc, Manassas, VA) that was supplemented with 25 mmol/L HEPES, L-glutamine, and 10% fetal bovine serum (tRPMI). The ectocervix and endocervix were cleansed with chlorhexidine solution (Hibiclens; Mölnlycke Health Care, Norcross, GA) and dried with a sterile swab. Endometrial aspiration biopsy specimens (Pipelle; CooperSurgical Inc) were obtained; care was taken not to touch the aspirator to the vaginal walls or the ectocervix. Adequacy of the sample was assessed visually by the clinician who was obtaining the biopsy. Endometrial samples were transported in the aspirator to the laboratory and extruded under sterile conditions. All samples were transported to the laboratory for processing within 30 minutes of collection.

Endometrial biopsy specimens were weighed and then washed 3-4 times with phosphate-buffered saline solution without calcium and magnesium (PBS; Mediatech Inc). The biopsy specimens were minced with the use of sterile scissors and placed in digest buffer that contained 20 mL RPMI-1640, 1 mg/mL collagenase D (Roche Ltd, Nutley, NJ), and 2000 U/mL DNase I (0.5 μL/mL; New England Biosciences, Ipswich, MA). Agitation of the tissue in digest buffer was limited to 15 minutes at approximately 300 rpm at 37°C to maintain cell surface marker integrity. The transport vial that contained the endocervical cytobrush was vortexed and washed with tRPMI to dislodge cells from the cytobrush. Both biopsy and cytobrush-collected cells were filtered through a 40-μm nylon cell sieve (Becton Dickenson, Franklin Lakes, NJ) to obtain single cell suspensions.

Endometrial biopsy specimens additionally underwent density gradient centrifugation to remove dead cells, red blood cells, and other debris. The digested and filtered endometrial cells were resuspended in 5 mL 36% Histopaque (Sigma-Aldrich, St. Louis, MO) in RPMI then layered over 4.5 mL undiluted Histopaque and under 500 μL PBS. This tube was centrifuged at 600g for 30 minutes with no brake; lymphocytes were recovered from the interface. Recovered cells of both specimen types were then washed by centrifugation in RPMI and resuspended in 1 mL PBS. Using Trypan blue stain (Sigma-Aldrich) exclusion criteria, viable cell yields were obtained manually with a hemocytometer.

Cell suspensions were adjusted to 1 x 10^6 cells/mL in PBS; 1 mL was stained for viability with LIVE/DEAD Fixable Aqua Dead Cell Stain (Invitrogen, Carlsbad, CA) and incubated for 25 minutes at room temperature protected from light. Cells were washed once with 1 mL of flow cytometry staining buffer (FACS; eBioscience, San Diego, CA) by centrifugation for 5 minutes at 400g and stained with fluorochrome-conjugated antibodies (BD Biosciences, San Jose, CA) specific for the following cell surface markers: CD45 (FITC), CD3 (PerCP), CD8 (APC-H7), CD4 (PacificBlue), CD195 (CCR5)(APC), CD69 (PE), and CD14 (PE-Cy7). Cells were incubated for 25 minutes at room temperature protected from light, washed with 1 mL FACS buffer by centrifugation for 5 minutes at 400g, and fixed in 1% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA). Stained samples were stored at 4°C, and flow cytometric analysis was conducted no later than 24 hours after fixation.

Lymphocyte populations were analyzed using an LSR II flow cytometer (BD Biosciences). To compensate for differences in cell density, the antibody concentrations were standardized to 30 μg/mL.

Figure 2: Consolidated Standards of Reporting Trials flow diagram

Trail profile. The diagram demonstrates numbers of participants who were screened, enrolled, randomly assigned, and analyzed in the trial.
spectral overlap, single color compensation was applied specific for each fluorochrome-conjugate used. T-cell populations were identified with the use of forward and side scatter, and fluorescent-minus-one controls were used to assist in defining gate positions. Two senior laboratory technicians who were trained in advanced flow cytometry independently reviewed and agreed on the gating parameters for each sample.

The sample size for this study was calculated based on available published data that indicated the mean percent expression of CCR5 on cervical CD4 cells among women not using contraception was 48 ± 4%, with a standard deviation of ±7% for women who used COCs. Assuming the standard deviation of the mean change in the percent expression would be no greater than ±8%, a sample size of 10 would have 90% power to detect at least a 20% difference (set to be >1 standard deviation for mean change) in the percent expression of CCR5 on CD4 measured before and 2 months after IUD placement, with the use of a paired t-test evaluated at the .05 2-sided significance level. The enrollment target was increased to 16 participants per group to account for potential loss to follow-up evaluation, postrandomization ineligibility, inadequate specimen quality, and the plan to evaluate additional cell populations.

Data were analyzed with FACS DIVA software (version 6.2; BD Biosciences) and FlowJo software (version 10.0.5; Tree Star Inc, Ashland, OR). The gating strategy for all populations can be seen in Figure 1. Cell numbers from biopsy specimens were normalized per gram of tissue. Cytobrush-collected cells were reported as “cells per cytobrush.” The cell numbers quantified were log_{10} transformed for analysis and presentation. Expression of CCR5 and CD69 was reported as the percentage of parent population that expressed these cell surface markers. Each participant served as her own control by the use of the baseline visit as the control normal and

<table>
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\(^a\) Data are presented as mean ± SD; \(^b\) One-way analysis of variance; \(^c\) Data are presented as median (min, max); \(^d\) Kruskal-Wallis test; \(^e\) Fisher exact test.

42 women were enrolled in the study between December 2010 and July 2011. Assessment of change at the 2-month follow-up visit. Statistical analysis was performed with SPSS statistical software (version 20.0; IBM Corporation, Armonk, NY), and statistical tests were evaluated at the 2-sided .05 significance level. Differences in enrollment characteristics between the groups were assessed with 1-way analysis of variance and Kruskal-Wallis and Fisher exact tests, where appropriate. Differences in levels of expression from baseline to follow-up were evaluated with paired Student t tests.

**RESULTS**

Between December 2010 and July 2011, 42 women were enrolled in the study (Figure 2). Demographic information was not significantly different among the groups, including phase of menstrual cycle at the time of enrollment (Table). All of the sample data from 1 participant who had been assigned randomly to the LNG-IUD group were excluded from analysis because of inadequate specimen quality after a tissue handling error. The endometrial biopsies were performed with a single pass for 78 of 82 biopsy specimens (95%). The remaining 4 biopsies required 2 attempts to obtain an adequate sample.

Two months after IUD insertion, there was no statistically significant change from baseline in the number of CD4+ or CD8+ T cells in the endometrium (Figure 3) or cervix (data not shown) among women who used hormonal and nonhormonal IUDs. The number of CD14+ immune cells (macrophages, neutrophils, and dendritic cells) in the endometrium significantly increased 2 months after Cu-IUD placement (log_{10} 3.4 → 4.3; P < .001); among women who received an LNG-IUD, the increase was less marked and not statistically significant (log_{10} 3.8 → 4.2; P = .06).

Within the endometrium, the percentage of CD4+ and CD8+ T cells that expressed the CCR5 HIV coreceptor significantly decreased from baseline levels 2 months after LNG-IUD insertion (66% → 34%; P < .001; and 72% → 41%; P < .005, respectively; Figure 4, A). CCR5 expression also significantly decreased from baseline levels on endometrial CD8+ T cells 2 months after Cu-IUD insertion (70% → 43%; P < .005).

Within the cervix, CCR5 coreceptor expression on endocervical CD4+ T cells was diminished significantly from baseline levels 2 months after initiation of an IUD (54% → 38%; P < .05 for LNG-IUD and 55% → 35%; P < .01 for Cu-IUD; Figure 4, B). There was a decrease, but nonsignificant, expression of the CCR5 coreceptor on cervical CD8+ T cells, compared with baseline in women who were assigned randomly to Cu-IUD (62% → 47%; P = .06).

T-cell activation was assessed by measurement of the percentage of T cells that expressed CD69. Endometrial cells from women who used the LNG-IUD had significantly decreased CD69 on CD8+ T cells (81% → 68%; P < .05) compared with baseline; there was also a nonsignificant decrease in activation of CD4+ T cells compared with baseline (67% → 52%; P = .06; Figure 4, A). In women who received a Cu-IUD, there was no significant change over 2 months in the activation of T cells within the endometrium; however, in the cervix, there was a significant decrease in the percentage of activated CD4+ and CD8+ T cells compared with baseline (66% → 47%; P < .01; and 76% → 58%; P < .05, respectively; Figure 4, B).

No significant changes were observed over time in any of these parameters among the control women who did not receive an IUD.

**COMMENT**

Although there is an incomplete understanding of factors that can increase or decrease CCR5 and CD69 expression on genital T cells, CCR5 expression appears to increase predominantly with viral and parasitic infections. Expression of both CD69 and CCR5 on various immune cells appear to be increased with exposure to proinflammatory cytokines and chemokines; such an inflammatory milieu in the genital tract has been associated prospectively with increased HIV acquisition risk. Conversely, CD69 and CCR5 expression on lymphocytes may be decreased with exposure to steroids and antibiotics. Based on suggestions that injectable progestins may increase susceptibility to HIV infection, we had hypothesized that local progestin delivery with an LNG-IUD would have a greater impact on genital immune cells compared with exposure to a nonhormonal Cu-IUD, altering HIV susceptibility. Surprisingly, there was no change in the number of T cells; the percentage of T cells that expressed HIV-coreceptor CCR5 was reduced, and the activation state of the T cells was either reduced or unchanged within the upper and lower genital tracts 2 months after the initiation of either a hormonal or nonhormonal IUD. No statistically significant T-cell changes, which included CCR5 and CD69 expression, occurred among women in the parallel control group. Taken together, these data suggest IUD use (either hormonal or copper) do not induce a proinflammatory milieu in the genital tract and would not increase HIV transmission risk.

We evaluated immune cell populations simultaneously in the upper and lower genital tracts of women who began IUD use to assess a range of sexually exposed mucosa because the primary site of sexual transmission remains unknown. Furthermore, the Cu-IUD has long been purported to create an...
inflammatory reaction within the endometrium as part of its mechanism of contraceptive action; however, we did not find evidence of such. We found no increase in the activation of T cells as measured by CD69 expression in the endometrium of IUD users. We did find a statistically significant increase in macrophages, neutrophils, and dendritic cells in the endometrium of Cu-IUD users as measured by CD14 and a similar, but not significant, increase in these cells among the LNG-IUD users. Interestingly, these data suggest differential alterations in immune cellular populations after Cu-IUD insertion, with an increase in innate immune cells (CD14+) and a decrease in lymphocyte activation. Importantly, innate immune cells at mucosal surfaces, particularly macrophages and dendritic cells, may also play a significant role in HIV transmission by capturing and transporting HIV virions to susceptible T cells.

There are few studies to date that have evaluated immune cells from freshly collected upper reproductive tract tissue surrounding IUD use. Studies that use in situ genital tract immune cells generally use tissue that is collected at surgery from heterogeneous patients who undergo procedures for a variety of pathologic conditions and with a mean age of >40 years. Given evidence that age modifies the relationship between contraceptive use and risk of HIV acquisition, with younger women at greater risk, biopsies rather than the use of surgical specimens allows investigation of the endometrial immune cells of younger healthy women. Because most studies that have evaluated genital tract immune cells to date have been performed with cytobrush-collected cervical cells, we also chose to include cytobrush-collected cells in the present study. More work is needed to better understand the correlation between cells that are recovered by brush compared with tissue biopsy cells.

To minimize the effects of natural variation in cellular populations over time and with respect to sexual practices, we used a pair-wise comparison study design such that women acted as their own controls. The 100% follow up of study participants in this study contributed to the strength of this analysis. One limitation of the present study is the single follow-up visit and the brief 2-month evaluation time. A strength of the present study was the inclusion of a control group of women who were observed in parallel, because this group of women had no statistically significant changes in immune cell populations over time, which suggests that the changes that were observed among the women who began IUD use was not due to normal variability of these cell populations over time.

Given the low probability of HIV transmission per sexual exposure to an HIV-infected partner, more research is needed to characterize the HIV target cells that are present in the female genital tract and how their numbers, activation status, and coreceptor expression relate to HIV susceptibility. Further studies are needed that directly compare
genital CD4+ T-cell subsets and antigen-presenting cells in women who begin the full range of hormonal and nonhormonal contraceptives, including DMPA, to better understand the range of cellular alterations and to learn which changes, if any, are important determinants of susceptibility to HIV. Furthermore, a better understanding of the endometrial and cervical immune effects of Cu-IUD use, particularly in the context of enrolling Cu-IUD users as nonhormonal contraceptive “controls” for larger trials that are designed to understand HIV risk with contraceptive use, are needed urgently.

In conclusion, this study of reproductive-aged women who were beginning IUD use demonstrated that women who used the LNG-IUD had decreased numbers of CD4 cells that expressed the HIV coreceptor CCR5, in both the endocervix and the endometrium, compared with baseline, suggested that the numbers of HIV targets in the cervix and endometrium would be decreased after the initiation of this hormonal IUD. Women who used the Cu-IUD had a similar decrease in CD4 cells that expressed the CCR5 receptor in the cervix, which suggests that use of either type of IUD is associated with changes in T-cell populations in the female genital tract that are not suggestive of an increased risk of HIV acquisition. Given that the HIV target cell populations were decreased largely in the genital tract with IUD use, a hypothesis could be generated that IUD use, particularly LNG-IUD use, may be somewhat protective for HIV acquisition. The direct effect of these IUD-induced changes on actual cellular susceptibility (either increased, decreased, or unchanged) has yet to be evaluated.

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