Long-term Effect of Depot Medroxyprogesterone Acetate on Vaginal Microbiota, Epithelial Thickness and HIV Target Cells

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Background. Depot medroxyprogesterone acetate (DMPA) has been linked to human immunodeficiency virus type 1 (HIV-1) acquisition.

Methods. Vaginal microbiota of women using DMPA for up to 2 years were cultured. Mucosal immune cell populations were measured by immunohistological staining.

Results. Over 12 months, the proportion with H2O2-positive lactobacilli decreased (n = 32; 53% vs 27%; P = .03). Median vaginal CD3+ cells also decreased (n = 15; 355 vs 237 cells/mm2; P = .03), as did CD3+CCR5+ cells (195 vs 128 cells/mm2; P = .04), HLA-DR+ cells (130 vs 96 cells/mm2; P = .27), and HLA-DR+CCR5+ cells (18 vs 10 cells/mm2; P = .33).

Conclusions. DMPA contraception does not increase vaginal mucosal CCR5+ HIV target cells but does decrease CD3+ T lymphocytes and vaginal H2O2-producing lactobacilli.

Keywords. HIV; medroxyprogesterone acetate; vaginal mucosa.

Over half of the 33 million people living with human immunodeficiency virus type 1 (HIV-1) in 2009 were women, most of whom were infected through heterosexual intercourse [1].

A possible link between HIV acquisition and use of depot medroxyprogesterone acetate (DMPA) has been described, although data vary significantly between studies [2]. DMPA is used for contraception by >26 million women worldwide [3]. Few prospective studies have assessed the risk of HIV acquisition in DMPA users directly, and given the significant risks associated with unintended pregnancy, the World Health Organization has not changed recommendations on the safety of DMPA [4]. However, because of the significant risk to public health if use of DMPA increases HIV acquisition, it is important to seek biologically plausible mechanisms for this association to aid in designing future studies and making policy choices. In this study, we examined the effect of DMPA on the following 3 potential biologic mechanisms for an association with HIV acquisition: HIV target cell abundance, vaginal microbiota, and epithelial thickness.

METHODS

Healthy women 18–40 years old desiring DMPA for contraception were enrolled in Seattle, Washington, and followed every 3 months for up to 2 years with collection of blood, vaginal swab specimens, and vaginal biopsy specimens at each visit, as previously described [5]. Vaginal biopsy specimens were fixed in 10% neutral buffered formalin (VWR) and embedded in paraffin. Blood was tested for estradiol, vaginal swab specimens underwent Gram staining, and vaginal bacteria were identified by routine culture methods [5]. The University of Washington Institutional Review Board approved the study protocol, and all subjects gave written, informed consent.

As previously described, epithelial layers were counted on hematoxylin and eosin–stained samples in 3 separate fields at 40 times the original magnification [5]. Periodic acid–Schiff stain was used to evaluate the epithelial cell glycogen content. In situ analysis of specific vaginal cell populations was performed in 2 stages. First, we performed immunohistochemical staining for CD1a (Langerhans cells) and CD4, as previously described [6]. Positive cells were manually counted by a single microscopist (D. P.) in 5 high-powered microscope fields (400× original magnification) per sample. Second, we performed immunofluorescence staining for the following 2 combinations of markers for HIV target cells: HLA-DR/CCR5 and CD3/CCR5. We analyzed digitized images, allowing for computer-aided analysis of stained sections, as recently described in detail elsewhere [7] and in the Supplementary Methods. On average, 11 101 cells were analyzed per slide (median, 10 552 cells/slide; range,
885–30 068 cells/slide). Cells were identified as positive if they fell above the positive-negative thresholds for both mean and maximum fluorescence intensity.

For these analyses we included only women who completed at least 12 months of follow-up. Statistical analyses for all end points except immunohistochemical and immunofluorescence-stained cell counts were done using linear and logistic mixed-effect models to account for correlated data. Median cell counts based on immunohistochemical and immunofluorescence staining were compared between enrollment and 1 year of DMPA, using a Wilcoxon signed rank test for paired data. All analyses were performed using Stata, version 11 (StataCorp).

RESULTS

Among 60 women screened, 58 received the first DMPA injection, and 32 of those women attended at least 12 months of study visits and are included in this analysis; 22 (69%) of those 32 women have data available for at least 1 outcome at 24 months. Participants were young (median age, 20 years, range, 18–39 years), mostly white (23/32; 72%), had never smoked (21/32; 66%), and had a median of 2.5 sex partners in their lifetime (range, 0–12 sex partners/lifetime). No participants received a diagnosis of gonorrhea, chlamydial infection, yeast infection, or trichomoniasis at enrollment.

The mean number of cell layers (±SD) did not change significantly over the course of DMPA use (27 ± 6 layers at baseline; 29 ± 5 layers after 12 months [P = .21, vs baseline; n = 29]; and 27 ± 4 layers after 24 months [P = .30 vs baseline; n = 16]). Epithelial thickness overall and glycogen-positive thickness trended slightly lower between baseline and 12 months, but this did not reach statistical significance (Table 1).

Over 12 months, the percentage of women with H2O2-positive Lactobacillus decreased significantly (from 53% to 27%; P = .03; n = 30; Table 1). The proportion of women with a diagnosis of bacterial vaginosis, based on Nugent score, did not change significantly over the first year, although there was a trend toward an increased number of women with an altered Nugent score (ie, a score of >3; 29% at enrollment vs 47% at 12 months; P = .08) and with a culture positive for Gardnerella vaginalis (19% at enrollment vs 33% at 12 months; P = .07; n = 30).

Ten women had bacterial cultures completed at the 24-month follow-up; the proportion with H2O2-positive Lactobacillus (40%) was only slightly lower than at enrollment (P = .56), and there were no significant differences in the proportion with an abnormal Nugent score or G. vaginalis (Table 1). However, the lack of statistical significance may be due to the small number of subjects with data after the 12-month time point.

The density of HIV target cells in the vagina was examined in 2 ways: (1) simple immunohistochemical analysis for cells expressing the HIV receptor CD4 and for CD1a+ Langerhans

| Table 1. Changes in Biologic Markers With Depot Medroxyprogesterone Acetate Use Over 12 and 24 Months |
|----------------|----------------|----------------|----------------|----------------|
| Marker                     | Enrollment     | Month 12       | P value        | Month 24       | P value        |
| Estradiol, mean ± SD       | n = 32         | n = 32         | .0003          | n = 9          |                |
| Estradiol level, pg/mL     | 118 ± 64       | 29 ± 13        | <.001          | 28 ± 12        | .003c          |
| Histology, mean ± SD       | n = 32         | n = 29         | .26            | n = 16         | .30            |
| Epithelial cell layers, no. | 27 ± 4         | 29 ± 5         | .21            | 27 ± 4         | .30            |
| Epithelial thickness, mm   | 1.02 ± 0.2     | 0.96 ± 0.25    | .26            | ND             | NA             |
| Glycogen-positive thickness, mm | 0.80 ± 0.24   | 0.74 ± 0.27    | .35            | ND             | NA             |
| Nugent score, % positive   | n = 32         | n = 30         | .08            | n = 10         | .68            |
| Altered flora (score >3)   | 29             | 47             | .54            | 10             | .96            |
| Bacterial vaginosis (score >6) | 13            | 17             |                |                |                |
| Bacterial cultures, % positive | n = 32        | n = 30         | .03            | n = 10         | .13            |
| H2O2-positive Lactobacillus | 53             | 27             | .07            | 40             | .79            |
| H2O2-negative Lactobacillus | 6             | 17             | .18            | 40             | .05            |
| Gardnerella vaginalis      | 19             | 33             |                | 20             | .92            |
| Ureaplasma urealyticum     | 56             | 50             | .66            | 50             | .08            |
| Prevotella species         | 34             | 37             | .80            | 30             | .07            |
| Anaerobic gram-positive cocci | 50           | 50             | .45            | 60             | .18            |
| Escherichia coli           | 25             | 23             | .61            | 20             | .92c           |
| Candida albicans           | 25             | 13             | .32            | 20             |                |

Abbreviations: ND, Not done; NA, not applicable.

a Data are for linear or logistic mixed-model analyses for the women who had follow-up at 12 months.
b Unless otherwise indicated, data are for a logistic mixed-model analysis including data from all 22 women who had follow-up after 12 months, of whom 10 had data at 24 months.
c Data are for a linear mixed model including all 24 women with more than 12 months of follow-up, of whom 16 had measurements at 24 months.
cells, specialized intraepithelial dendritic cells that can bind and internalize HIV; and (2) immunofluorescence combination staining of the HIV coreceptor CCR5 with either CD3 (for T cells) or HLA-DR (targeting HLA-DR\textsubscript{high} dendritic cells, including Langerhans cells). By simple immunohistochemical analysis, the median number of CD1a\textsuperscript{+} Langerhans cells per 5 high-powered fields (400x original magnification) was 5 at enrollment (range, 0–50 cells/5 high-powered fields) and decreased significantly to 0 cells/5 high-powered fields (range, 0–20 cells/5 high-powered fields) at 12 months ($P = .04$; $n = 32$). Likewise, the median CD4\textsuperscript{+} T-cell count decreased from enrollment (15 cells; range, 0–85 cells) to 12 months (5 cells; range, 0–125 cells; $P = .02$; $n = 31$).

For combination immunofluorescence, we stained vaginal biopsy specimens collected at baseline and 12 months from 15 women (Figure 1). The median number of CD3\textsuperscript{+} T cells decreased significantly between baseline and 12 months, from 355 cells/mm\textsuperscript{2} (mean [±SD], 499 ± 463 cells/mm\textsuperscript{2}) to 237 cells/mm\textsuperscript{2} (mean [±SD], 280 ± 179 cells/mm\textsuperscript{2}; $P = .03$; Figure 1A). The number of HLA-DR\textsuperscript{+} cells trended down, from a median of 130 cells/mm\textsuperscript{2} (mean [±SD], 153 ± 91 cells/mm\textsuperscript{2}) to 96 cells/mm\textsuperscript{2} (mean [±SD], 153 ± 190 cells/mm\textsuperscript{2}; $P = .27$; Figure 1A). The median number of CCR5\textsuperscript{+} cells also trended down over 1 year of DMPA use, from 395 cells/mm\textsuperscript{2} (mean [±SD], 461 ± 402 cells/mm\textsuperscript{2}) to 270 cells/mm\textsuperscript{2} (mean [±SD], 303 ± 160 cells/mm\textsuperscript{2}; $P = .17$; Figure 1A). The median number of double-positive CD3\textsuperscript{+}CCR5\textsuperscript{+} T cells decreased significantly, from 195 cells/mm\textsuperscript{2} (mean, 280 ± 278 cells/mm\textsuperscript{2}) to 128 cells/mm\textsuperscript{2} (mean, 149 ± 97 cells/mm\textsuperscript{2}; $P = .04$; Figure 1B). However, the percentage of CCR5\textsuperscript{+} T cells among all CD3\textsuperscript{+} T cells remained nearly stable, with a median of 65% (mean [±SD], 58% ± 14%) before and 54% (mean [±SD], 56% ± 10%) after 12 months of DMPA use ($P = .48$) (Figure 1B). Similarly, the median number double-positive HLA-DR\textsuperscript{+}CCR5\textsuperscript{+} cells trended down, from 18 cells/mm\textsuperscript{2} (mean [±SD], 26 ± 24 cells/mm\textsuperscript{2}) to 10 cells/mm\textsuperscript{2} (mean, 20 ± 20 cells/mm\textsuperscript{2}; $P = .34$; Figure 1C), but the median percentage of CCR5\textsuperscript{+} cells among all HLA-DR\textsuperscript{+} cells remained stable, at 16% (mean [±SD], 15% ± 8%), compared with 12% (mean [±SD], 16% ± 10%; $P = .76$; Figure 1C).

These immunohistological data provide no evidence for an

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**Figure 1.** Summary of CD3\textsuperscript{+}, HLA-DR\textsuperscript{+}, and CCR5\textsuperscript{+} cell densities and percentages in all vaginal biopsy specimens analyzed at baseline and after 12 months of depot medroxyprogesterone acetate (DMPA) use. A, Densities of CD3\textsuperscript{+} cells (brown; left panel), HLA-DR\textsuperscript{+} cells (blue; middle panel), and CCR5\textsuperscript{+} cells (red; right panel). B, Densities of dual-positive CD3/CCR5-expressing cells (left y-axis) and percentages of CD3\textsuperscript{+} T cells coexpressing CCR5 (right y-axis) in 15 women. C, Densities of dual-positive HLA-DR/CCR5-expressing cells (left y-axis) and percentages of HLA-DR\textsuperscript{+} T cells coexpressing CCR5 (right y-axis) in 17 women. The horizontal line in each box denotes the median, the ends of the box denote the interquartile range, and the whiskers indicate the range. Paired samples (ie, samples in which data were derived from before and after 12 months of DMPA therapy in the same woman) are connected by lines. Decreasing cell densities or percentages are indicated by dotted lines; others are indicated by solid lines. Paired Wilcoxon signed rank test $P$-values for differences between baseline and 12 months are listed above the corresponding boxes.
increase of CCR5+ HIV target cell densities in the vaginal mucosa caused by DMPA contraception. Rather, extended DMPA use led to a substantial decrease in the number of mucosal CD3+ T cells.

**DISCUSSION**

Epidemiological studies suggest that DMPA increases susceptibility to HIV infection [2]. Efforts to define the mechanism(s) for this observation have so far focused on in vitro experiments [8], animal models [9], and short-term (duration, <6 months) effects of DMPA in women [10, 11]. Our study is the first to evaluate a longer-term (duration, up to 24 months) effect of DMPA use on the human vaginal immune system. By digitized in situ analysis of over approximately 10 000 cells in each tissue section, we found a decrease in CD3+ T cells and no change in HLA-DR+ antigen-presenting cells in the vaginal mucosa 1 year of DMPA use. In addition, expression of the HIV coreceptor CCR5 was unaffected, as measured by staining with the only anti-CCR5 antibody that reliably works in formalin-fixed tissues [7]. Thus, DMPA use does not appear to enhance HIV susceptibility by increasing the presence of HIV target cells in the vagina.

Moreover, we confirmed prior results that DMPA does not thin the vaginal epithelium in women [5, 10, 11], a finding that stands in stark contrast to the strong vaginal thinning effect of high-dose progesterone observed in macaques [9, 12]. Thus, we can exclude vaginal thinning as an underlying cause of increased HIV acquisition in women using DMPA. In addition, these findings illustrate the limitations of using animal models to mimic the effects of human sex hormones and underscore the need to perform such mechanistic investigations in humans.

However, DMPA produced other consequences on the vaginal environment. Over 1 year of use, the percentage of women with vaginal H2O2-producing lactobacilli colonization nearly halved, and the number of CD3+ T cells infiltrating the vaginal mucosa decreased by one-third. While we cannot draw a direct link from these changes to HIV susceptibility, these data raise some concerns regarding DMPA’s effect on the immune defenses of the mucosa. A recent study found that cervical exfoliants collected from women in the secretory phase of the menstrual cycle (a time of high progesterone) were significantly more likely to be infected by HIV ex vivo than tissue collected during the proliferative phase [13]. Fewer vaginal T cells, in conjunction with their reported impairment in proliferative and cytokine-producing capacity [8], could lower the mucosa’s adaptive immune response to infections, including HIV. Of note, although the density of CD3+ T cells was reduced by DMPA, our data indicate that the remaining cells retain baseline levels of CCR5 expression and thus can likely serve as targets for HIV infection. Moreover, DMPA appears to enhance HIV replication in T cells in vitro [8]. This state of vulnerability may be worsened by suppression of innate immune defenses by DMPA [8] and reduction in colonization with H2O2-producing lactobacilli, which have been associated with lower rates of HIV acquisition [14].

Although we present important data on the effects of long-term use of DMPA, our study is limited by the small number of women who returned for follow-up at 24 months, which may bias findings at later time points or diminish our power to find small but important differences in markers of interest. Additionally, while the present study provides important insights into the longer-term effects of DMPA on the human vaginal mucosa, these findings cannot be extrapolated to other female genital mucosae. It is conceivable that DMPA modulates immunity at the ectocervix, the endocervix, or the endometrium differently than in the vagina. It also is unknown whether DMPA can impact the rectal mucosa and anal HIV transmission. Likewise, the effect of DMPA may not be uniform during the 3 months following each injection. Peak progesterone levels are achieved approximately 8 days after injection [15] and then slowly wane over the remaining 80 days before the next injection. Analogous to the window of vulnerability to HIV infection that is thought to exist in the secretory phase of the menstrual cycle, a window of enhanced vulnerability could also exist at a certain period after DMPA injection. The samples for our study were taken only at the end of each 3-month cycle, when the progesterone concentration was at its lowest. Future studies should address the effects of DMPA on a wider spectrum of anatomical sites and during more narrowly spaced time points, including at peak progesterone levels.

In summary, the vaginal immune system is clearly impacted by longer-term DMPA contraception. The density of CD3+ T cells decreases, which could be a consequence of inhibition of lymphocyte-attracting chemokine production [8] or of diminished T-cell proliferation [8]. Colonization by H2O2-producing lactobacilli decreases, the mechanism of which requires further investigation. On the other hand, CCR5 expression on individual T cells and epithelial thickness appear unaffected. These findings suggest that DMPA causes a complex set of events that could on balance raise HIV susceptibility, but not by simply increasing target cell access or densities.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyrighted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

**Acknowledgments.** We thank Kimberly R. Melton and Julie Randolph-Habecker, for assistance with immunofluorescence staining of vaginal biopsies; Sean Hughes and Stephen Voght, for critical reading and editing of the manuscript; and the study participants, for volunteering for this study.
Disclaimer. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Financial support. This work was supported by the National Institute of Child Health and Human Development and the National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH); awards R01HD033203 [to F. H.] and UM1AI068618 [to M. J. M.]; and the NIAID, NIH (award 1K08AI087969-01 to C. M. M.).

Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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