Progesterone-Induced Inhibition of Chemokine Receptor Expression on Peripheral Blood Mononuclear Cells Correlates with Reduced HIV-1 Infectability In Vitro

Neratzoula Vassiliadou, Lynne Tucker and Deborah J. Anderson

*J Immunol* 1999; 162:7510-7518; 
http://www.jimmunol.org/content/162/12/7510
Progesterone-Induced Inhibition of Chemokine Receptor Expression on Peripheral Blood Mononuclear Cells Correlates with Reduced HIV-1 Infectability In Vitro

Neratzoula Vassiliadou, Lynne Tucker, and Deborah J. Anderson

Recent studies have shown that progesterone, a sex steroid hormone, enhances the sexual transmission of various pathogens, including SIV. The goal of this study was to determine whether progesterone affects mechanisms underlying the sexual transmission of HIV-1. We first studied the effects of various physiologic concentrations of progesterone on the expression of chemokines and chemokine receptors by T cells and macrophages. Chemokines are involved in leukocyte recruitment to peripheral sites; in addition, the chemokine receptors CCR5 and CXCR4 are HIV-1 coreceptors, and their ligands can block HIV-1 infection. Progesterone treatment had no effect on constitutive expression of CCR5 and CXCR4 by nonactivated T cells and macrophages, but significantly inhibited IL-2-induced up-regulation of CCR5 and CXCR4 on activated T cells (p < 0.05). Progesterone also inhibited both mitogen-induced proliferation and chemokine secretion (macrophage inflammatory protein-1α, MIP-1α, and MIP-1β), mediates entry of macrophage-tropic (M-tropic) HIV-1 strains (5, 6, 8–10). The importance of CCR5 has been underscored by reports of resistance to HIV-1 infection of individuals who are homozygous for a nonfunctional CCR5 allele that contains a 32-bp coding region deletion (Δ32) (11–14). CCR5 is expressed on cells in mucosal epithelia and is thought to be the most important chemokine receptor mediating HIV-1 transmission across mucosal surfaces (15–17).

Progesterone is a sex steroid hormone, naturally produced in the ovary and placenta. High levels are detected in serum of women during the secretory phase of the menstrual cycle and pregnancy. A bidirectional interrelationship between sex steroids and the immune system has been established over the years (18–21). Progesterone influences immune responses, including macrophage phagocytosis, NK cell activity, CTL activity, T cell proliferation, and the secretion of Th1-type cytokines (24–27). On the other hand, cytokines produced by lymphocytes and macrophages can regulate progesterone secretion by placental trophoblast and ovarian granulosa cells (28–30). The cellular and molecular mechanisms by which sex steroids manipulate immune responses are incompletely understood. Recent evidence indicates that human CD8+ T cells, but not CD4+ cells, express estrogen receptors (31). Human lymphocytes and monocytes apparently do not express the classical progesterone receptor (32, 33); they do, however, express the glucocorticoid receptor, which has a distinct progesterone binding domain (34).

It has been known for some time that animal models of sexually transmitted diseases require priming with progesterone (35–37). It was recently shown that rhesus macaques with s.c. progesterone implants were more susceptible to SIV vaginal transmission (38). It has been postulated that enhanced infection of the female genital tract after progesterone treatment is due to thinning of the vaginal epithelium.
Materials and Methods

Study design

Healthy women (n = 8) with normal menstrual cycles, between 22–36 yr of age, and not taking hormonal contraceptives were recruited for the study. All the protocols involving use of human material were approved by the Brigham and Women’s Hospital institutional review board. To standardize progesterone effects in our experiments, peripheral blood was obtained during the early proliferative phase of the menstrual cycle (days 6–8), when circulating progesterone levels are lowest. Plasma was separated from heparinized blood and stored at −70°C until tested to determine estrogen and progesterone levels. The hormone assays were performed at the Department of Reproductive Endocrinology, Massachusetts General Hospital (Charlestown, MA), using the IMx estradiol assay and the Axsym progesterone assay (Abbott Laboratories, North Chicago, IL). PBMCs were isolated from heparinized blood by Ficoll-Hypaque density gradient centrifugation (Amersham, Piscataway, NJ). Serum from each individual was also prepared from nonheparinized whole blood using standard techniques and was stored at −20°C until use.

All study participants were genotyped by PCR (see below) to determine whether they contained the Δ32 mutant CCR5 allele. This allele has a profound role in resistance to HIV-1 acquisition by PBMCs, and recent studies have also indicated that +/Δ32 and Δ32/Δ32 individuals express markedly reduced levels of CCR5 that cannot be up-regulated even after IL-2 treatment (39); only individuals homozygous for the wild-type CCR5 allele were included in the study.

Progesterone (Sigma, St. Louis, MO) was used at 10−3, 10−6, and 10−9 M final concentrations. These concentrations were chosen because they can be found in different physiologic states: 10−7 M corresponds to levels present in the circulation during the secretory phase of the cycle (40), and 10−6 M corresponds to peripheral blood levels during pregnancy (41), and concentrations of progesterone as high as 10−3 M have been detected at the fetal-maternal interface (42).

RNA isolation and RT-PCR analysis

Total RNA was extracted from PBMCs using Trizol reagent (Life Technologies, Grand Island, NY), according to the manufacturer’s instructions. One microgram of RNA was treated with 1 U/μl DNase I (Life Technologies) for 15 min at room temperature to eliminate genomic DNA. Following DNase inactivation (addition of 1 M final concentration of EGTA), RNA integrity was assessed on a 1.2% agarose gel in the presence of 0.25 g/ml ethidium bromide. PCR-ELISA detection

All reagents were purchased from Boehringer Mannheim, and the assay was performed according to the manufacturer’s instructions. Briefly, 10 μl of DIG-labeled PCR samples were incubated in duplicate with 20 μl of denaturation buffer; the single DNA strands were then hybridized with specific biotin-labeled probes (5′-3′, b-AAGATTTCCAGACATTAAAGATGTCATCTC; CCRX4, b-CTCAGTGAGCCAGATGCAGATA; TAT, GAPDH, b-CCCTAGGCACAGTGCAGCT) and incubated for 2 h at 35°C in avidin-coated plates. Plates were then washed three times, incubated with anti-DIG peroxidase-conjugated Ab (10 μg/ml; 30 min at 37°C), washed, and further incubated with 2,2′-azino-bis(3-ethylbenzthiazol)-6-sulfonic acid (ABTS) substrate (30 min at 37°C). The absorbance was read at 405 nm. The PCR negative control as well as blank wells, in which the PCR product was replaced with wash buffer, defined the absorbance due to nonspecific binding. The value obtained from the blank wells was subtracted from all samples. The resulting OD readings were presented as CCR5 or CCRX4 OD/OD GAPDH OD ratios to normalize the results obtained for the chemokine receptor mRNA with results from a housekeeping gene from the same RNA preparation.

PCR-ELISA detection

All reagents were purchased from Boehringer Mannheim, and the assay was performed according to the manufacturer’s instructions. Briefly, 10 μl of DIG-labeled PCR samples were incubated in duplicate with 20 μl of denaturation buffer; the single DNA strands were then hybridized with specific biotin-labeled probes (5′-3′, b-AAGATTTCCAGACATTAAAGATGTCATCTC; CCRX4, b-CTCAGTGAGCCAGATGCAGATA; TAT, GAPDH, b-CCCTAGGCACAGTGCAGCT) and incubated for 2 h at 35°C in avidin-coated plates. Plates were then washed three times, incubated with anti-DIG peroxidase-conjugated Ab (10 μg/ml; 30 min at 37°C), washed, and further incubated with 2,2′-azino-bis(3-ethylbenzthiazol)-6-sulfonic acid (ABTS) substrate (30 min at 37°C). The absorbance was read at 405 nm. The PCR negative control as well as blank wells, in which the PCR product was replaced with wash buffer, defined the absorbance due to nonspecific binding. The value obtained from the blank wells was subtracted from all samples. The resulting OD readings were presented as CCR5 or CCRX4 OD/OD GAPDH OD ratios to normalize the results obtained for the chemokine receptor mRNA with results from a housekeeping gene from the same RNA preparation.

Progesterone effects on constitutive CCR5 and CCRX4 expression in nonactivated T cells

PBMCs were cultured at a cell density of 106/ml in AIM V phenol red-free and serum-free medium (Life Technologies) or phenol red-free RPMI supplemented with 1000 U/ml penicillin, 1 mg/ml streptomycin, 2 mM L-glutamine, 0.1 mM MEM nonessential amino acids (all purchased from Fisher Scientific, Springfield, NJ), and 10% autologous serum. Progesterone (10−8 M) was added at the initiation of the cultures; nonadherent cells were harvested from progesterone and control (medium alone) wells at 24 and 72 h for CCR5 and CCRX4 quantification.

Progesterone effects on IL-2-induced up-regulation of CCR5 in activated T cells

The method of Wu et al. (39) was followed to up-regulate CCR5 protein expression in PBMCs. Blasts were generated using anti-CD3 (2 μl/106 cells; Ortho Diagnostics, Raritan, NJ) in AIM V medium; after 4 days, nonadherent cells were transferred to fresh RPMI medium supplemented with antibiotics, 10% autologous serum, and 100 U/ml recombinant human IL-2 (Becton Dickinson, San Jose, CA). Cells from each individual were split into two groups. In group A cells were cultured with progesterone at three different final concentrations (10−5, 10−6, and 10−7 M) from day 1 of culture, whereas in group B cells were cultured with progesterone at various time points after CCR5 up-regulation had started and also when maximum up-regulation had been achieved for varying periods of time (24, 48, and 72 h). Cells were maintained in culture for up to 4 wk. Parallel cultures without progesterone served as negative controls for each sample.

Progesterone effects on IL-2-induced up-regulation of CCRX4 in activated T cells

Cells (106/ml in AIM V medium) were cultured in the presence of PHA (Boehringer Mannheim; final concentration, 1.2 μg/ml) and progesterone at three different final concentrations (10−5, 10−6, and 10−7 M). After 3 days cells were fed with fresh RPMI supplemented with antibiotics, 10% autologous serum, and IL-2 (200 U/ml). Investigation of CCRX4 protein expression in nonadherent PBMCs started on days 3–4 and continued up to days 9–10. Cells cultured in the absence of progesterone served as negative controls for each sample.

Progesterone effects on constitutive CCR5 and CCRX4 expression in monocytes/macrophages

PBMCs were cultured for 3–4 days in AIM V medium or RPMI supplemented with antibiotics and 10% autologous serum in the presence of the absence of progesterone (10−8 and 10−7 M). Following culture, nonadherent cells were removed, and culture wells were treated with 0.5 mM EDTA (two times; 10 min each time) to detach the adherent macrophage-enriched cell population.

mAbs and FACS analysis

mAbs specific for CCR5 (5C7, LeukoSite, Cambridge, MA) (39) and CCRX4 (12G5, J. Hoxie) (43) were obtained through the AIDS Research
and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health (Bethesda, MD), CD3-P (Coulter, Hialeah, FL), CD4-P, CD8-P, and CD14-P (Becton Dickinson) mAbs were also used. Cells (0.4 × 10^6) were resuspended in PBS, pH 7.4, containing 2% heat-inactivated human serum (Scantibodies Laboratory, Santee, CA), 0.1% sodium azide, and 10 μg/ml CR5 or CXCR4 mAbs. After 25 min at 4°C, cells were washed twice in wash buffer (PBS, pH 7.4, containing 0.2% BSA and 0.1% sodium azide) and incubated for 20 min in the dark with goat anti-mouse Ig conjugated with FITC (Becton Dickinson). Following a brief wash in buffer, cells were analyzed on the FACScan (single labeling) or were incubated with a second mAb directly conjugated with PE for 20 min at 4°C, washed, and then analyzed on the FACScan (double labeling) using CellQuest software (Becton Dickinson). Propidium iodide was used to exclude dead cells.

Analysis of CXCR4 expression was also performed in whole blood samples. Briefly, 100 μl of blood was incubated with CXCR4 mAb (20 min, room temperature). RBC were lysed using Pharm Lyse (PharMingen, San Diego, CA). Following RBC lysis, samples were washed twice, incubated with FITC-labeled goat anti-mouse Ig, and analyzed in the FACScan as detailed above.

For each sample, 10,000 cells were analyzed. Isotype-matched mAb controls (Sigma), used at the same concentration as the mAbs, served as negative controls to determine the amount of fluorescence due to nonspecific binding.

**Immunofluorescence staining for confocal microscopy**

Fluorescence intensity ofCXCR4 cell surface expression in the progesterone-treated PBMC cultures was also assessed using confocal microscopy. Cells were maintained for 4–5 days in the presence of PHA with and without progesterone (10−7 M). On day 3 cells were fed with RPMI supplemented with antibiotics, 10% autologous serum, and IL-2 as detailed above. Following incubation, cells were prepared for confocal imaging according to the method of Amara et al. (44). Briefly, cells were fixed in 3.7% paraformaldehyde in PBS for 20 min, washed in PBS, incubated with 0.1 M glycine in PBS to quench free aldehydes, permeabilized with 0.05% saponin in PBS supplemented with 0.2% BSA for 15 min, and sequentially stained with CXCR4 mAb and FITC-conjugated goat anti-mouse Ig as described. Following staining, cells were mounted in Vectashield mounting medium (Vector, Burlingame, CA) and viewed in a Leica TCSNT confocal laser scanning microscope (Leica, Exton, PA). Fluorescence confocal micrographs were recorded by exposing selected fields of view to 488 nm light. A 510-nm long-pass filter was used to select light emitted from the fluorochrome. ImageSpace software (Molecular Dynamics, Sunnyvale, CA) was used to measure the average pixel intensity of individual cells; an average of three fields were analyzed per sample.

**Progesterone effects on chemokine secretion by activated T cells**

CD4+ and CD8+ T cells were purified using the MidiMACS magnetic separation system (Miltenyi Biotec, Auburn, CA). Briefly, PBMCs were incubated with CD4 or CD8 MACS microbeads for 15 min at 4°C, and the magnetically labeled cell suspension was passed through a MidiMACS separation system (Miltenyi Biotec, Auburn, CA). PBMCs were incubated with CD4 or CD8 MACS microbeads for 15 min at 4°C, washed, and then analyzed on the FACScan (single labeling) or were incubated with a second mAb directly conjugated with PE for 20 min at 4°C, washed, and then analyzed on the FACScan (double labeling) using CellQuest software (Becton Dickinson). Propidium iodide was used to exclude dead cells.

Analysis of CXCR4 expression was also performed in whole blood samples. Briefly, 100 μl of blood was incubated with CXCR4 mAb (20 min, room temperature). RBC were lysed using Pharm Lyse (PharMingen, San Diego, CA). Following RBC lysis, samples were washed twice, incubated with FITC-labeled goat anti-mouse Ig, and analyzed in the FACScan as detailed above.

For each sample, 10,000 cells were analyzed. Isotype-matched mAb controls (Sigma), used at the same concentration as the mAbs, served as negative controls to determine the amount of fluorescence due to nonspecific binding.

**Immunofluorescence staining for confocal microscopy**

Fluorescence intensity of CXCR4 cell surface expression in the progesterone-treated PBMC cultures was also assessed using confocal microscopy. Cells were maintained for 4–5 days in the presence of PHA with and without progesterone (10−7 M). On day 3 cells were fed with RPMI supplemented with antibiotics, 10% autologous serum, and IL-2 as detailed above. Following incubation, cells were prepared for confocal imaging according to the method of Amara et al. (44). Briefly, cells were fixed in 3.7% paraformaldehyde in PBS for 20 min, washed in PBS, incubated with 0.1 M glycine in PBS to quench free aldehydes, permeabilized with 0.05% saponin in PBS supplemented with 0.2% BSA for 15 min, and sequentially stained with CXCR4 mAb and FITC-conjugated goat anti-mouse Ig as described. Following staining, cells were mounted in Vectashield mounting medium (Vector, Burlingame, CA) and viewed in a Leica TCSNT confocal laser scanning microscope (Leica, Exton, PA). Fluorescence confocal micrographs were recorded by exposing selected fields of view to 488 nm light. A 510-nm long-pass filter was used to select light emitted from the fluorochrome. ImageSpace software (Molecular Dynamics, Sunnyvale, CA) was used to measure the average pixel intensity of individual cells; an average of three fields were analyzed per sample.

**Progesterone effects on chemokine secretion by activated T cells**

CD4+ and CD8+ T cells were purified using the MidiMACS magnetic separation system (Miltenyi Biotec, Auburn, CA). Briefly, PBMCs were incubated with CD4 or CD8 MACS microbeads for 15 min at 4°C, and the magnetically labeled cell suspension was passed through a MidiMACS separation column. Positive cells remained attached to the column and the magnetic labeled cell suspension was passed through a MidiMACS separation system (Miltenyi Biotec, Auburn, CA). Briefly, cells were fixed in 3.7% paraformaldehyde in PBS for 20 min, washed in PBS, incubated with 0.1 M glycine in PBS to quench free aldehydes, permeabilized with 0.05% saponin in PBS supplemented with 0.2% BSA for 15 min, and sequentially stained with CXCR4 mAb and FITC-conjugated goat anti-mouse Ig as described. Following staining, cells were mounted in Vectashield mounting medium (Vector, Burlingame, CA) and viewed in a Leica TCSNT confocal laser scanning microscope (Leica, Exton, PA). Fluorescence confocal micrographs were recorded by exposing selected fields of view to 488 nm light. A 510-nm long-pass filter was used to select light emitted from the fluorochrome. ImageSpace software (Molecular Dynamics, Sunnyvale, CA) was used to measure the average pixel intensity of individual cells; an average of three fields were analyzed per sample.

**Progesterone effects on chemokine secretion by activated T cells**

CD4+ and CD8+ T cells were purified using the MidiMACS magnetic separation system (Miltenyi Biotec, Auburn, CA). Briefly, PBMCs were incubated with CD4 or CD8 MACS microbeads for 15 min at 4°C, and the magnetically labeled cell suspension was passed through a MidiMACS separation column. Positive cells remained attached to the column and were recovered using a plunger supplied with the column. Purified cells (10^6/ml) were cultured in AIM V medium in the presence of PHA with and without progesterone (10−7 M). Irradiated (5000 rad) allogeneic macrophages (adherent cells remaining after adding 10^6 irradiated PBMCs) were also present in the CD8+ T cell cultures. Cell-free supernatants were collected at the termination of the culture period (40). Following incubation, cells were prepared for confocal imaging according to the method of Amara et al. (44). Briefly, cells were fixed in 3.7% paraformaldehyde in PBS for 20 min, washed in PBS, incubated with 0.1 M glycine in PBS to quench free aldehydes, permeabilized with 0.05% saponin in PBS supplemented with 0.2% BSA for 15 min, and sequentially stained with CXCR4 mAb and FITC-conjugated goat anti-mouse Ig as described. Following staining, cells were mounted in Vectashield mounting medium (Vector, Burlingame, CA) and viewed in a Leica TCSNT confocal laser scanning microscope (Leica, Exton, PA). Fluorescence confocal micrographs were recorded by exposing selected fields of view to 488 nm light. A 510-nm long-pass filter was used to select light emitted from the fluorochrome. ImageSpace software (Molecular Dynamics, Sunnyvale, CA) was used to measure the average pixel intensity of individual cells; an average of three fields were analyzed per sample.
analysis or by double labeling using CXCR4 or CCR5 mAbs and PE-conjugated anti-CD4 or anti-CD8.

**CCR5.** Addition of IL-2 led to marked CCR5 protein up-regulation in control cultures. Investigation of the effect of progesterone started as soon as up-regulation was detected. Progesterone, added on day 1 of culture and remaining for the entire culture period (group A), significantly suppressed CCR5 protein expression at both $10^{-5}$ M ($p < 0.001$) and $10^{-6}$ M ($p < 0.05$; Fig. 2, a and b). Although the time point at which peak CCR5 up-regulation occurred and the proportion of CCR5$^+$ cells detected varied among individuals, progesterone treatment ($10^{-5}$ M) resulted in at least a 35% reduction in the proportion of CCR5-expressing T cells (range, 35–87%; Fig. 2a). This progesterone-mediated effect could be seen only after IL-2-induced CCR5 up-regulation; when progesterone-treated and control cultures from each individual were tested for CCR5 expression before CCR5 up-regulation had started (days 7–10; Fig. 2a), they both demonstrated a similar percentage of CCR5$^+$ cells, in agreement with the results obtained regarding the effect of progesterone on the constitutive CCR5 protein expression. There was no significant suppression of CCR5 expression when progesterone was added to the cultures at $10^{-7}$ M (Fig. 2b), except for one individual who showed consistent suppression even with the $10^{-7}$ M concentration. Interestingly, this individual had the highest percentage of CCR5$^+$ cells in control cultures after IL-2-induced up-regulation. Double labeling studies demonstrated that both CD4$^+$ and CD8$^+$ subpopulations expressed CCR5, and that progesterone inhibited CCR5 expression to the same extent in both subpopulations. Progesterone treatment did not affect CD4 or CD8 Ag expression (data not shown). The mean fluorescence intensity (MFI) of the CCR5 signal in positive cells was also significantly decreased in progesterone-treated ($10^{-5}$ M) cultures vs

**FIGURE 2.** *A,* Effect of progesterone on CCR5 protein expression in activated T cells. Each graph represents an individual case ($n = 6$). Progesterone ($10^{-5}$ M; dotted line), added on day 1 of culture and remaining for the entire culture period, significantly suppressed CCR5 protein up-regulation in anti-CD3/IL-2-activated cells in all cases studied and at all time points compared with that in the control (no progesterone; solid line) cultures ($p < 0.001$). *B,* Dose-response curve of the progesterone effect on CCR5 surface expression in anti-CD3/IL-2-activated T cells. Effects of different progesterone concentrations on CCR5 expression were studied at the time of peak CCR5 expression in nontreated control cultures (days 14–23). Progesterone significantly suppressed IL-2-induced CCR5 up-regulation at both $10^{-5}$ M ($p < 0.001$) and $10^{-6}$ M ($p < 0.05$), whereas there was no significant suppression of CCR5 protein expression at $10^{-7}$ M. Results are presented as the mean percentage of CCR5$^+$ cells ± SEM ($n = 6$ individuals).
CONTROL OF HIV-1 INFECTION MECHANISMS

FIGURE 3. MFI of CCR5 in control and progesterone (10^{-5} M)-treated peripheral blood cell cultures from six individuals. T cells were activated with anti-CD3 in the presence and the absence of progesterone. CCR5 protein expression was up-regulated with IL-2 (100 U/ml). Each point in the graph represents the value obtained for a single individual. The reduction of MFI in progesterone-treated positive cells ranged from 35 to 87% compared with that in the control untreated cultures at times of peak CCR5 expression (days 14–23). The decrease in MFI values in the progesterone-treated cultures compared with control values was significant (p < 0.01). To calculate MFI, the positively stained cells were gated, based on background values of isotype-matched negative controls, and MFI was calculated using CellQuest software.

CXCR4. PHA activation and subsequent IL-2 addition resulted in CXCR4 up-regulation. CXCR4 protein expression was studied at a minimum of three time points for each individual, starting from days 3 to 4 of culture. Peak CXCR4 protein expression in PHA/IL-2-activated T lymphocytes was detected between days 5–8. All three concentrations of progesterone caused a significant reduction in the number of PBMCs expressing CXCR4 (p < 0.001; Fig. 4a).

The MFI of CXCR4 cells was also significantly reduced in the progesterone-treated cultures at both 10^{-5} and 10^{-6} M compared with that in the control cell cultures (mean MFI ± SEM: control, 13.22 ± 1.33; progesterone-treated, 5.94 ± 1.41; p < 0.01; Fig. 3). Semiquantitative analysis of mRNA expression at the same time points used for protein studies revealed a significant up-regulation in CCR5 mRNA levels in anti-CD3/IL-2-activated PBMCs compared with nonactivated cells (p < 0.01). However, progesterone had no effect on CCR5 mRNA expression levels in activated T cells (data not shown).

Incubation of PBMCs with progesterone (10^{-5} and 10^{-7} M) for varying periods of time (24, 48, and 72 h) during CCR5 up-regulation and when maximum CCR5 expression had been achieved (group B) had no effect on CCR5 mRNA expression (data not shown).

FIGURE 4. A. Effects of different concentrations of progesterone on CXCR4 protein expression in PHA/IL-2-activated T cells. Cells were incubated with progesterone at three different concentrations and analyzed after 5–7 days in culture. The maximum suppressive effect on CXCR4 coreceptor expression was noted at the highest hormone concentration used, at which there was at least a 42% reduction in the proportion of CXCR4^{+} cells in progesterone-treated cultures compared with that in the control untreated cultures (range, 42–68%). Progesterone also significantly suppressed CXCR4 protein expression at the lower concentrations (10^{-6} and 10^{-7} M; p < 0.001). B. MFI of CXCR4^{+} PHA/IL-2-activated T cells, analyzed by confocal laser scanning microscopy, in control (A) and progesterone-treated (B) cultures. The fluorescence intensity of the CXCR4^{+} progesterone-treated cells was at least 50% reduced compared with that of the control untreated cells (mean of three independent experiments; p = 0.04).

Progesterone effects on constitutive chemokine receptor expression in monocytes/macrophages

Expression of CCR5 and CXCR4 by the adherent monocyte/macrophage population after 3–4 days in culture was investigated by double labeling with cells with CXCR4 or CCR5 mAbs and PE-conjugated CD14 and setting the gates on monocytes/macrophages. CCR5. There was heterogeneity among individuals in the proportions that CCR5^{+} CD14^{+} cells formed in relation to either the gated cell population or the CD14^{+} cell population (mean percentage of CCR5^{+} CD14^{+} cells: range, 5.05–34.14%; n = 3). Nevertheless, within each sample, treatment with progesterone, even at the maximum 10^{-5} M concentration, did not alter the proportion of CCR5^{+} CD14^{+} cells.

CXCR4. Progesterone did not affect expression of CXCR4 in monocytes/macrophages at any of the concentrations tested (mean...
Progesterone effects on chemokine secretion by activated T lymphocytes

CD4\(^+\) cells secreted significantly more MIP-1\(\alpha\) than MIP-1\(\beta\) and RANTES \((p < 0.04)\), whereas there was no significant difference among the chemokine secretion levels in CD8\(^+\) cells (Fig. 5, a and b). CD4\(^+\) T cells secreted significantly more MIP-1\(\alpha\) than did CD8\(^+\) T cells \((p = 0.028)\).

Progesterone at the maximum concentration significantly inhibited the secretion of all three chemokines by CD8\(^+\) cells \((p < 0.04)\), whereas it had no effect on chemokine secretion by CD4\(^+\) cells (Fig. 5, a and b). To determine whether the reduced chemokine secretion by progesterone-treated CD8\(^+\) cells was due to a negative effect of progesterone on cell proliferation, we tested the proliferative responses of both CD4\(^+\) and CD8\(^+\) T cells to PHA in the presence and the absence of progesterone. Progesterone significantly suppressed the proliferation of CD8\(^+\) cells \((\text{mean} \pm \text{SEM}: \text{control}, 123,311 \pm 30,227 \text{ cpm}; \text{progesterone-treated}, 72,149 \pm 19,061 \text{ cpm}; p = 0.013)\), whereas it did not significantly affect the proliferation of CD4\(^+\) T cells \((\text{control}, 91,104 \pm 17,849; \text{progesterone-treated}, 77,615 \pm 13,574; p = 0.16)\). Since chemokine levels were reduced by 50–70% in progesterone-treated CD8\(^+\) cell cultures (Fig. 5b), and the mean counts per minute value of CD8\(^+\) progesterone-treated cells was 58% that of untreated cells, it is possible that the suppressive effect of progesterone on chemokine secretion by CD8\(^+\) cell cultures was due to the inhibition of cell proliferation and the lower cell numbers in these cultures.

**Discussion**

Considerable research effort has been directed toward the identification and development of molecules that can prevent or regulate HIV-1 infection. It is well established that adrenal steroid hormones (glyocorticosteroids) promote HIV-1 infection via activation of a steroid response element in the HIV long terminal repeat region \((49, 50)\). Preliminary studies provide evidence that other steroid hormones, such as estrogens and progesterone, also modulate HIV infection in vitro \((51, 52)\). However, given the complexity of the interactions between the immune and endocrine systems and limited understanding at the molecular level of sex hormone-lymphocyte interactions, the roles of these hormones in HIV-1 infection are unclear. The present study, using an in vitro system, addressed whether progesterone influences HIV-1 infection mechanisms via effects on the chemokine-chemokine receptor system.

In nonactivated PBMCs, CCR5 expression remained low and was unaffected by progesterone treatment. Addition of IL-2 following anti-CD3 activation of T cells resulted in marked up-regulation of CCR5 mRNA and protein expression, consistent with previous reports \((39, 53)\). Progesterone exerted a dose-dependent
inhibitory effect on CCR5 protein expression in these activated cultures. This inhibitory effect of the hormone could be seen only when progesterone was added before cell activation and was present for the entire culture period; CCR5 expression was unaltered when progesterone was added after activation of the cells, including the time of peak CCR5 expression.

In contrast to CCR5, CXCR4 protein expression was rapidly and markedly up-regulated in nonactivated PBMCs. This noninduced up-regulation of CXCR4 in freshly isolated peripheral blood T cells suggests that expression of this receptor may be negatively regulated in situ. Treatment with progesterone had no effect on CXCR4 mRNA or protein expression in these cells. On the other hand, progesterone suppressed IL-2-induced CXCR4 protein up-regulation in PHA-activated PBMCs even at the lowest (10⁻⁷ M) concentration; both the percentage of CXCR4-positive cells and the level of CXCR4 per positive cell were significantly reduced in progesterone-treated cultures.

No significant differences were detected between control and progesterone-treated cultures when mRNA levels for CXCR4 and CCR5 were measured by semiquantitative PCR. It is possible that the method employed was not sensitive enough to detect differences between control and test samples. Alternatively, it is possible that progesterone effects occur at the post-transcriptional level.

Progesterone suppressed IL-2-induced CCR5 and CXCR4 protein expression in PHA-activated PBMCs. The suppressive effect of progesterone on CCR5 and CXCR4 protein expression, seen exclusively in activated T cells and not in resting T cells or macrophages, suggests that the hormone interferes with early cellular activation events. However, preliminary studies of other early activation markers, such as CD69 and IL-2R α- and β-chain expression in PHA-activated cells, indicate that progesterone does not affect de novo expression and/or up-regulation of these molecules (N. Vassiliadou and D. J. Anderson, unpublished observations). This raises the possibility that progesterone targets the chemokine/chemokine receptor gene system directly.

The effect of progesterone on chemokine receptor protein expression could have implications for lymphocyte migration. Chemokines direct movement of leukocytes in development, homeostasis, and inflammation via interactions with their receptors (54). Chemokines, actively produced at sites of inflammatory processes, attract T cells and monocytes among other cell types and have been implicated in the development of both acute and chronic inflammatory conditions (55). Decreased expression of chemokine receptors could result in altered patterns of cell migration and secretion of cytokines and other inflammatory and immune mediators.

Progesterone treatment resulted in significantly lower concentrations of RANTES, MIP-1α, and MIP-1β in supernatants from CD8⁺ T lymphocyte cultures, whereas it did not affect concentrations of these chemokines in CD4⁺ T cell cultures. Considering that progesterone selectively inhibited the proliferation of CD8⁺ T cells, it is possible that the lower concentrations of chemokines in progesterone-treated CD8⁺ cell cultures were due to inhibition of cell proliferation by the hormone. CD8⁺ cells predominate in the epithelial layer of mucosal epithelia and are thought to serve important mucosal immune defense functions (56). Our data suggest that progesterone may inhibit chemokine-mediated recruitment and proliferation of activated CD8⁺ T lymphocytes and could thereby weaken mucosal antimicrobial defense functions. On the other hand, by reducing chemokine secretion by activated CD8⁺ T cells at sites of infection, progesterone treatment could result in a
reduction in the number of CD4+ cells recruited by chemokines to the mucosal epithelium, thereby decreasing the number of HIV-1 host cells at sites of transmission.

Considering the effects of progesterone in the chemokine-chemokine receptor system, it was of interest to directly assess the effects of progesterone on HIV-1 infection in vitro. Treatment with progesterone dramatically and consistently inhibited infection of activated PBMCs with low titers of the HIV-1MN T-tropic strain, indicating that the effect of the hormone in this model system was protective against HIV-1 infection. Since T cell-tropic viruses primarily use the CXCR4 coreceptor for entry, and CXCR4 expression (but not CD4 expression) was reduced in progesterone-treated cultures at the time of HIV-1 inoculation, our study suggests that progesterone may inhibit transmission of T-tropic strains of virus by decreasing CXCR4 expression on CD4+ host T cells. Our preliminary experiments using an M-tropic HIV strain also suggested that progesterone may inhibit infection of activated T cells with CCR5-tropic strains of virus by decreasing CCR5 expression.

Enhanced transmission of SIV and other sexually transmitted diseases in progesterone-treated experimental animals may be due to significant thinning of the vaginal epithelium (38, 57) and/or suppression of cytotoxic T cell and NK cell antiviral functions. Recent studies in women indicate that progesterone does not affect vaginal epithelial thickness to the same extent (D. J. Anderson et al., unpublished observations); furthermore, our data suggest that progesterone suppresses the expression of chemokine receptors in activated lymphocytes and inhibits HIV-1 infection by T-tropic and M-tropic viral strains in vitro. This information and meta-analyses of recent epidemiologic studies of HIV-1 acquisition in women on progestin-based contraceptives (58) suggest that progesterone does not enhance HIV-1 infection in women. More research is needed at the molecular level to further elucidate the effects of progesterone on mechanisms of viral entry and intracellular events leading to down-regulation of chemokine receptors. Furthermore, it will be important to determine whether progesterone-associated changes in chemokine-chemokine receptor profiles observed in PBMCs correlate with altered expression of these molecules at mucosal sites and affect patterns of T cell homing and T cell/monocyte recruitment to sites of infection.

Acknowledgments

We thank A. J. Quayle, Ph.D., and P. A. Crowley-Nowick, Ph.D., for their helpful comments during the performance of the work and the writing of the manuscript, and J. A. Polich, Ph.D., for help with the statistical analysis of the data.

References


