Infection of Macrophages and Dendritic Cells with Primary R5-Tropic Human Immunodeficiency Virus Type 1 Inhibited by Natural Polyreactive Anti-CCR5 Antibodies Purified from Cervicovaginal Secretions

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Heterosexual contact is the primary mode of human immunodeficiency virus (HIV) type 1 (HIV-1) transmission worldwide. The chemokine receptor CCR5 is the major coreceptor that is associated with the mucosal transmission of R5-tropic HIV-1 during sexual intercourse. The CCR5 molecule is thus a target for antibody-based therapeutic strategies aimed at blocking HIV-1 entry into cells. We have previously demonstrated that polyreactive natural antibodies (NAbs) from therapeutic preparations of immunoglobulin G and from human breast milk contain NAbs directed against CCR5. Such antibodies inhibit the infection of human macrophages and T lymphocytes by R5-tropic isolates of HIV in vitro. In the present study, we demonstrate that human immunoglobulins from the cervicovaginal secretions of HIV-seronegative or HIV-seropositive women contain NAbs directed against the HIV-1 coreceptor CCR5. Natural affinity-purified anti-CCR5 antibodies bound to CCR5 expressed on macrophages and dendritic cells and further inhibited the infection of macrophages and dendritic cells with primary and laboratory-adapted R5-tropic HIV but not with X4-tropic HIV. Natural anti-CCR5 antibodies moderately inhibited R5-tropic HIV transfer from monocyte-derived dendritic cells to autologous T cells. Our results suggest that mucosal anti-CCR5 antibodies from healthy immunocompetent donors may hamper the penetration of HIV and may be suitable for use in the development of novel passive immunotherapy regimens in specific clinical settings of HIV infection.

The female genital tract possesses various systems of defense against the risk of infection, and these appear to be complementary and even synergistic (4, 28). Innate defense involves the humoral immune response, with secretory immunoglobulin A (S-IgA), secretory IgM (S-IgM), and locally produced IgG (s-IgG) able to activate a strong cellular immune response (4, 23). The very large amount of IgG in female genital tract secretions, which occurs at levels more than 10-fold those of IgA and which originate in part from plasma by transudation, is remarkable for a corporeal fluid, whereas mucosal secretions are most often characterized by the predominance of the IgA isotype (2, 4, 23).

Natural antibodies (NAbs) are produced by B-1 cells, irrespective of any immunization procedure, and thus belong to the innate immune system (20, 32). In contrast to antigen-primed antibodies, these low-affinity antibodies are polyreactive and may recognize different unrelated epitopes and autoantigens (1, 3, 20). As evidenced by the study of purified human monoclonal paraproteins, NAbs recognize both self-antigens and microbial antigens (1, 3, 9). Several functions have been proposed for polyreactive NAbs, including defense against pathogens (especially in the first immune barrier against microorganisms in the digestive tract), immunoregulation, and immune clearance of autoantigens (1, 3, 32). A pathological role for NAbs has been suggested in autoimmune, tumor cell recognition, and atherosclerosis (3, 9, 20). It has been shown that human colostrum and saliva contain large amounts of S-IgA polyreactive NAbs (44). The natural humoral immunity of the female genital tract, however, has not yet been studied. The NAbs lining the female genital mucosa may also hamper the penetration of a pathogen just before defenses are acquired, and NAbs specific for the pathogen may be involved (3, 4, 32).

Heterosexual contact is the primary mode of human immunodeficiency virus (HIV) type 1 (HIV-1) transmission worldwide. The majority of new HIV-1-infected people are women.
The seven-transmembrane G-protein-coupled chemokine receptor CCR5 is one of the major coreceptors of HIV (17) and is associated with the mucosal transmission of R5-tropic HIV-1 (R5-HIV-1) during sexual intercourse (37, 49, 53). Indeed, CCR5 is involved in the entry of HIV-1 into its target cells (19), such as macrophages (51, 52), dendritic cells (10, 27, 52), and some CD4+ T-cell subsets (43), especially in the female genital tract (22, 35). The predominant role of CCR5 in HIV transmission was also demonstrated by the protective role of the Δ32 allele of CCR5 against HIV in homozgyotes (24, 34, 41, 48). The CCR5 molecule is thus a target for novel therapeutic strategies aimed at blocking the entry of HIV-1 into cells (26, 29, 45, 46, 47, 50). We have previously demonstrated that NAbs from therapeutic preparations of IgG (intravenous [i.v.] Ig) and from human breast milk contain NAbs directed against CCR5; such antibodies inhibit infection of human macrophages and T lymphocytes by R5-HIV isolates in vitro (12, 13).

We demonstrate here that cervicovaginal secretions contain large amounts of polyreactive NAbs, mainly of the IgG and S-IgA isotypes and, to a lesser extent, of the IgM isotype. Among these NAbs, we identified anti-CCR5 NAbs that are able to bind to CCR5 expressed on monococyte-derived macrophages (MDMs) and monocyte-derived dendritic cells (MDDCs). Affinity-purified anti-CCR5 NAbs further inhibited the infection of both MDMs and MDDCs by primary and laboratory-adapted R5-HIV without inhibiting infection induced by X4-HIV. In addition, anti-CCR5 NAbs moderately inhibited the transfer of R5-HIV from MDDCs to autologous T cells and faintly inhibited the transfer of X4-HIV particles.

MATERIALS AND METHODS

Patients. Female sex workers aged 18 years or older underwent a gynecological examination and routine microbiological tests for sexually transmitted infections at the Centre National de Référence des Maladies Sexuellement Transmissibles of Bangui, Central African Republic. Women were informed about the study, and oral consent was obtained, according to local ethical guidelines. The patients included in the study (n = 40) were HIV seronegative, were clinically asymptomatic (CD4 categories A1 and A2), and had not received any treatment for sexually transmitted diseases and who were not menstruating.

Detection of NAB activities by ELISA. Each antigen (bovine actin, porcine myosin, porcine thyroglobulin, human transferrin, or double-stranded human DNA) at 10 μg/ml was coated onto a 96-well plate (Maxisorb; Nunc) at 100 μl per well. After an extensive wash with PBS, the plates were incubated with PBS-1% milk at 37°C for 1 h. Serial twofold dilutions in PBS of the cellular fraction of the CVL fluid samples, i.e., IgG, or IgM myeloma (100 μg/ml) were further incubated in each coated well at 37°C for 1 h. Afterwards, the activities were revealed with horseradish-peroxidase-labeled biotin-conjugated mouse Ig to the human Fab fragment, Feε, and Feγ. For the detection of natural IgM, biotin-conjugated mouse Ig Feγ was used after incubation with HRP-labeled second antibody. Peroxidase activity was revealed with o-phenylenediamine, and the optical density (OD) was read at 492 nm. For each assay, dilution, and isotype, the cutoffs for positivity were determined as the mean OD plus 2 standard deviations (SDs) obtained from 15 acellular fractions of PSA-negative cervicovaginal secretions from HIV-seronegative women from whom IgG, IgM, and IgG were previously absorbed with polyclonal anti-human Feε, Feγ, and Feγ (sheep) antibody, respectively, with further precipitation with 3.75% (wt/vol) polyethylene glycol 6000 (Sigma Chemical Co., St. Louis, MO), as described previously (15). The concentrations of total IgG, IgA, IgC, and IgM in the cervicovaginal secretions were quantified by a sandwich ELISA, with a pool of sera from healthy individuals serving as a standard (14). The specific activities (A) of the antibodies in the cervicovaginal secretions to a given antigen were evaluated as the first dilution (1/d) that gave an OD between 0.5 and 1.5 (the region where the OD curve is approximately linear) and per μg of total IgG in the CVL fluid, respectively, according to the formula A = (OD × d/[Ig]) (in μg/ml), as described previously (42). Similarly, the specific activities of S-IgA, s-IgG, and S-IgM to a given antigen in the CVL fluid per μg of total IgA, IgG, and IgM, respectively, in the CVL fluid were calculated.

Detection of anti-CCR5 antibodies in CVL fluid. The anti-CCR5 activities in the cervicovaginal secretions were assessed by ELISA. Plates were coated with the CCR5 peptide (10 μg/ml) in PBS at pH 7.4 overnight at 4°C. The plates were washed with PBS-0.1% Tween prior to saturation with PBS-1% skim milk. Dilutions of the CVL fluid supernatants, i.e., Ig, or IgG myeloma to be tested were then added and the plates were incubated overnight at 4°C. After the plates
were washed, peroxidase-labeled goat anti-human F(ab')2 antibodies were added at 37°C for 1 h before addition of substrate.

**Immunofluorescence analysis of anti-CCR5 antibodies from vaginal secretions.** The CCR5 peptide was coupled to activated Sepharose 4B (Pharmacia Biotech) according to the manufacturer's instructions. Pools of cervicovaginal secretions were allowed to interact with the matrix at 4°C overnight before the column was extensively washed with PBS until the OD of the effluent reached 0.001. The column was then eluted with 0.2 M glycine-HCl, pH 2.5. The pH of the eluted material was rapidly neutralized with 3 M Tris, and the eluted material was further dialyzed against PBS overnight. The affinity-purified anti-CCR5 Ig that bound to the CCR5 peptide was assessed by an ELISA and revealed with anti-F(ab')2 antibodies. Immunofluorescence-purified anti-CCR5 antibodies had no activity against gp120 by the indirect ELISA.

The effluents were also collected, and the CCR5-depleted total IgGs within the effluents were subsequently purified with anti-human F(ab')2 antibodies coupled to activated Sepharose 4B at 4°C overnight. Following extensive washing of the column with PBS until the OD of the effluent reached 0.001, the column was then eluted with 0.2 M glycine-HCl, pH 2.5. The pH of the eluted material was rapidly neutralized with 3 M Tris, and the eluted material was further dialyzed against PBS overnight.

**Specificity of anti-CCR5 NAbs.** Plates were coated with the CCR5 peptide (10 μg/ml) in PBS at pH 7.4 and 4°C overnight. The plates were washed with PBS-0.1% Tween prior to saturation with PBS-1% skim milk. Anti-CCR5 NAbs purified from the CVL fluid from HIV-seronegative women (samples 3 and 7) and HIV-seropositive women (samples 23 and 26) were further added in the presence or the absence of increasing amounts of CCR5 peptide or anti-F(ab')2 antibodies at 4°C overnight. After the plates were washed, peroxidase-labeled goat anti-human F(ab')2 antibodies were added at 37°C for 1 h before addition of substrate. Percent inhibition was defined as the ratio between the OD value obtained in the presence of CCR5 peptide or anti-F(ab')2 antibodies and the control OD value obtained without inhibitors.

**Avidity of anti-CCR5 antibodies from vaginal secretions.** The avidity of anti-CCR5 NAbs isolated from HIV-seronegative women (samples 2 and 14) and HIV-seropositive women (samples 24 and 28) was assessed by the method of Pulendran et al. (40). Briefly, purified anti-CCR5 NAbs were incubated with the CCR5 peptide for 2 h at 37°C before treatment of the wells with increasing amounts of potassium thiocyanate (KSCN; 0.1 M to 3 M) for 30 min at room temperature. The wells were washed and incubated with goat anti-human F(ab')2 coupled to peroxidase for 1 h at 37°C. The molarity of KSCN required for 50% of the bound antibodies was then determined. The avidity index was defined as the molarity of KSCN equivalent to the interopelling point corresponding to 50% of the control absorbance value obtained in the absence of KSCN.

**MDM and MDDC preparation.** Peripheral blood mononuclear cells were isolated from the freshly drawn blood of healthy donors by Ficoll-Paque gradient centrifugation. The CD14+ fraction was purified from the peripheral blood mononuclear cells with magnetic beads (Miltenyi Biotec, Germany). Purified CD14+ MDMs contained less than 1% CD3+ T cells. The CD3+ T cells were subsequently frozen until use. MDMs were obtained following 7 days of culture of the monocytes in the presence of 10 ng/ml of macrophage colony-stimulating factor. At the time of collection, macrophages expressed CD14 (90%) and CD11b (90%). MDDCs were obtained after 6 days of culture in the presence of a combination of granulocyte-macrophage colony-stimulating factor and IL-4 at 10 ng/ml. On day 6, nonadherent mononuclear cells exhibited an immature dendritic cell phenotype (CD14+ CD1a+ DC-SIGN+ CD83+ low).

**Binding of purified anti-CCR5 peptide antibodies to primary CCR5-positive cells.** For binding experiments with purified anti-CCR5 antibodies, MDMs at day 7 of culture, immature MDDCs at day 6 of culture, and thawed T cells (10⁶ cells) were incubated with anti-CD14, anti-CD1a, and anti-CD3 MAbs, respectively, and with either purified anti-CCR5 NAbs (0, 100, or 1,000 μg/ml), anti-CCR5-depleted antibodies (1,000 μg/ml), or anti-CCR5 MAb (clone 2D7; 10 μg) at 4°C for 1 h. After the washes, the cells were incubated with FITC-conjugated goat anti-human Ig antibodies and anti-CCR5 PE MAb at 4°C for 30 min. The cells were subsequently incubated with FITC-conjugated goat anti-human Ig antibodies and anti-CCR5 PE MAb at 4°C for 30 min. The cells were then fixed with 1% paraformaldehyde and were analyzed by cytometry with a FACScalibur apparatus and Cell Quest software. The percentage of positive cells is indicated in quadrants defined according to the relevant isotype control.

**Inhibition of HIV-1 infection by antibodies to CCR5.** MDMs and MDDCs (10⁵ cells) were infected in 96-well plates with 1 ng/ml of HIV1NL-4.3, HIV1NL-8, or HIV1-BaL, respectively, for 7 days. The cells were washed and then cultured for 6 days. In some experiments, the cells were preincubated with purified anti-CCR5 CVL fluid NAbs (50, 500, or 1,000 μg/ml) or with 1,000 μg/ml of the corresponding amounts of anti-CCR5-depleted Ig, used as a negative control before virus addition. As a positive control for the inhibition of HIV infection, anti-CCR5 or anti-CXCR4 MAb (10 μg/ml) were added to the cells prior to infection with HIV. The HIV p24 antigen concentrations in the supernatants were measured by ELISA at day 6 postinfection.

To study the transfer of positive HIV to T cells, MDDCs were washed after infection and autologous PHA- and IL-2-stimulated lymphocytes (in a 1/5 ratio) were added. The HIV p24 antigen concentrations in the supernatants were measured by ELISA at day 3 postinfection.

**RESULTS**

**NAb reactivity in human cervicovaginal secretions.** The presence of NAbs was first evaluated in purified Ig fractions of pooled cervicovaginal secretions from HIV-seronegative and HIV-seropositive women. As depicted in Fig. 1, the total Ig (IgA, IgG, and IgM) purified from the CVL fluid samples from HIV-seronegative women reacted in a dose-dependent manner against bovine actin, double-stranded human DNA, porcine myosin, porcine thyroglobulin, and human transferrin, as did i.v. Ig, which was used as a positive control. As expected, no reaction of irrelevant myeloma antibodies was observed, whatever antigen was used (Fig. 1). Similarly, Ig purified from CVL fluid samples from HIV-seropositive women reacted in a dose-dependent manner against all antigens tested (Fig. 1). There was no significant difference in the reactivity intensities between pools from HIV-seronegative and HIV-seropositive women, except for actin and myosin with a high antibody concentration (500 μg/ml). No difference in NAb reactivities according to the presence or absence of traces of semen within the pools of the cervicovaginal secretions from either HIV-seropositive or HIV-seronegative patients was detected (data not shown).

CVL fluid antibodies reacting with bovine actin, double-stranded human DNA, porcine myosin, porcine thyroglobulin, and human transferrin could be detected by indirect ELISA (i.e., they gave ODs above the calculated threshold) with 6, 5, 6, 10, and 9 μg/ml of purified total CVL fluid Ig, respectively (Fig. 1).

The specificities of these purified NAbs from HIV-seronegative donors were also determined by adding increasing concentrations of i.v. Ig or soluble bovine actin (data not shown). We observed that the inhibition of i.v. Ig binding to bovine actin increased in an i.v. Ig dose-dependent manner.

Taken together, these observations demonstrate that Igs from the CVL fluid of either HIV-seropositive or HIV-seronegative women contained polyreactive NAbs.

**Total IgA, IgG, and IgM concentrations in CVL fluid samples.** We then characterized the concentrations of total IgA, IgG, and IgM present in pooled CVL fluid samples from either HIV-seropositive or HIV-seronegative women. Thus, the quantification of the concentrations of IgG, IgA, and IgM was performed. In the HIV-seronegative women, IgG and IgA were the most abundant Igs in pooled CVL fluid samples (37 and 36 μg/ml, respectively), as reported in our previous study (5). It is noteworthy that all IgA antibodies contained the secretory portion (data not shown). IgM antibodies were weakly detected (4 μg/ml) in pooled CVL fluid samples from women not infected with HIV. It is noteworthy that no signif-
significant difference between the fractions was observed in samples with or without traces of semen (data not shown).

However, the levels of IgG, IgA, and IgM measured in pooled CVL fluid samples from HIV-seropositive women (169, 66, and 14 μg/ml, respectively) were higher than the levels of IgG, IgA, and IgM in pooled CVL fluid samples from HIV-seronegative donors. As was reported for the HIV-seronegative women, no significant difference between CVL fluid samples from HIV-infected women with or without traces of semen was observed (data not shown).

Specific activities of NAb within CVL fluid samples. The specific activities of the IgA, IgG, and IgM antibodies to self-antigens were further evaluated, as presented in Table 1. For the HIV-seronegative women, the antibodies within each IgA, IgG, and IgM subclass reacted similarly whatever autoantigens were used. Among all Ig isotypes tested, IgM exhibited stronger specific activities toward autoantigens than IgA and IgG. No significant difference in the specific activities of IgA and IgG toward any of the autoantigens except actin was observed. In the presence or the absence of traces of semen, no signifi-

![FIG. 1. Autoantibody reactivities to bovine actin, double-stranded human DNA, porcine myosin, porcine thyroglobulin, and human transferrin in total Ig fractions purified from pooled cervicovaginal secretions. Antigens were coated on 96-well plates; and serial dilutions of i.v. Ig (open circles), IgG myeloma (bold diamonds), or pooled cervicovaginal secretions from HIV-seronegative women (HIV−; open triangles) or HIV-seropositive women (HIV+; bold squares) were further added, as described in Materials and Methods. The concentrations of the antigen-reacting antibodies were evaluated by the addition of biotin-conjugated mouse Ig against anti-human Fab, followed by the addition of streptavidin-peroxidase, and the results were revealed by substrate addition. The total Ig content (IgA, IgG, and IgM) of the preparations is shown on the abscissa. The results are expressed as the means of the values obtained from three independent experiments ± SDs. The reactivity intensities for actin and myosin between pools from HIV-seronegative and HIV-seropositive women were significantly different (P < 0.01) only for high antibody concentrations (500 μg/ml); *, P ≤ 0.01 by the paired Student t test between the specific activities of CVL fluid samples from HIV-seronegative and HIV-seropositive women.](image-url)

| Antigen       | HIV-seronegative women (n = 22) | HIV-seropositive women (n = 24) |
|---------------|---------------------------------|---------------------------------|
|               | AlgA  | AlgG  | AlgM  | AlgA  | AlgG  | AlgM  |
| Actin         | 0.07  | 0.70  | 3.40  | 3.08  | 1.17  | 15.42 |
| DNA           | 0.80  | 1.05  | 2.96  | 6.50  | 0.81  | 15.72 |
| Myosin        | 0.75  | 0.82  | 4.00  | 5.51  | 1.05  | 15.36 |
| Thyroglobulin | 0.55  | 0.94  | 4.16  | 3.34  | 0.45  | 9.96  |
| Transferrin   | 0.46  | 0.36  | 3.80  | 2.67  | 1.17  | 11.10 |

* Autoantibody reactivities were detected by an indirect ELISA and were revealed with biotin-conjugated mouse Ig against human Fc according to their isotypes (IgA, IgG, and IgM), as described in Materials and Methods.
* Specific activities in arbitrary units.
* The specific activities of antibodies of the IgA (AlgA), IgG (AlgG), or IgM (AlgM) isotype in CVL fluid to a given antigen were evaluated as the first dilution (1/d) that gave an OD between 0.5 and 1.5 and per μg of total IgA, IgG, or IgM, respectively, in the CVL fluid according to the formula A = (OD × d)/[Ig] (in μg/ml), as described previously (3, 40).
* The specific activities of the IgA and IgM NAb in CVL fluid were higher in HIV-seronegative women than in HIV-seropositive women (P < 0.02).
* The specific activities of IgG NAb in CVL fluid were similar in HIV-seronegative and HIV-seropositive women.
In HIV-positive women, IgM possessed stronger activity toward autoantigens than IgA and IgG, with IgG having the weakest activity. Similar to the results for the CVL fluid samples from HIV-seronegative women, significant differences in the specific activities toward the autoantigens were observed between CVL fluid samples with or without traces of semen only for thyroglobulin and transferrin-reactive IgA, which exhibited dramatic decreases in the presence of traces of semen (data not shown).

Interestingly, the specific activities of IgA and IgM were stronger in CVL fluid samples from HIV-seropositive women than in those from HIV-seronegative women. Conversely, the specific activities of IgG were not significantly different between pooled CVL fluid samples from either HIV-seronegative or HIV-seropositive women (Table 1).

NAbs to CCR5 in CVL fluid samples. As reported previously for NAbs from breast milk (13) or i.v. Ig (12), we further aimed at characterizing whether NAbs from CVL fluid contain CCR5-reacting NAbs. Therefore, the reactivities of Ig purified from pools of semen-free or semen-contaminated cervicovaginal secretions from HIV-negative and HIV-positive women were further evaluated against a relevant CCR5 peptide (12, 13). As shown in Fig. 2A, total Ig isolated from CVL fluid samples from either HIV-infected women or women not infected with HIV reacted against the CCR5 peptide in a dose-dependent manner, independently of the presence or the absence of traces of semen (data not shown). As expected, CCR5-reacting NAbs were detected in i.v. Ig. Conversely, no anti-CCR5 IgG was detected within irrelevant IgG from myelomas.

We then characterized the isotypic patterns of the CCR5-reactive NAbs present in CVL fluid samples from either HIV-positive or HIV-negative women with or without traces of semen (Fig. 2B). Similar to the other autoantigen-reactive NAbs, CCR5-reactive NAbs were of the IgA, IgM, and IgG isotypes. The isotypic patterns of the antibodies isolated from HIV-seronegative women showed that IgM exhibited stronger specific activity toward CCR5 than IgA and IgG did (Fig. 2B). The specific activities of anti-CCR5 IgA and anti-CCR5 IgM antibodies were significantly higher in CVL fluid samples from HIV-seropositive women than in CVL fluid samples from HIV-seronegative women. Conversely, no significant difference in the specific activities of anti-CCR5 IgG antibodies was observed between CVL fluid samples from HIV-seronegative women and those from HIV-seropositive women (Fig. 2B). No major difference in specific activity for CCR5 was observed between NAbs from CVL fluid samples with and without traces of semen (data not shown).

We further investigated CCR5-reacting NAbs at the individual level. CVL fluid samples from 18 HIV-seronegative women and 10 HIV-seropositive women were thus individually investigated for the presence of CCR5-specific antibodies. Sixty-one and 100% of the CVL fluid samples from HIV-seronegative and HIV-seropositive women, respectively, contained anti-CCR5 antibodies (Fig. 3). Nevertheless, this difference was not significant ($P = 0.21$).

Since false positivity may be obtained for corporeal fluids tested for the presence of specific antibodies, we confirmed that the reactivities to CCR5 observed were associated with the presence of anti-CCR5 antibodies by demonstrating that the ELISA reactivities obtained for four semen-free CVL fluid samples from two HIV-seronegative women (samples 3 and 7) and two HIV-seropositive women (samples 23 and 26) were inhibited by excess CCR5 peptide and anti-human F(ab')2 antibody in a dose-dependent fashion (Fig. 4A and B). The CCR5-specific antibodies purified from the CVL fluid samples from women 3, 7, and 26 were not fully competed by the CCR5 peptide CSSHPFYOSQYQFWKNFQTLK, which corresponds to the second extracellular loop of CCR5 (ILEC/C-CCR5), was coated on a 96-well plate; and serial dilution of i.v. Ig (open circles), IgG myeloma (bold diamonds), or pooled cervicovaginal secretions from HIV-seronegative women (HIV−; open triangles) or HIV-seropositive women (HIV+; bold square) were further added, as described in Materials and Methods. The concentration of CCR5-reacting antibodies was evaluated by the addition of biotin-conjugated mouse Ig against anti-human Fab, followed by the addition of streptavidin-peroxidase, and the results were revealed by substrate addition. The total Ig content (IgA, IgG, and IgM) of the preparations is shown on the abscissa. The results are expressed as the means of the values obtained from three independent experiments ± SDs. (B). Specific activities, in arbitrary units of IgA, IgG, and IgM NAbs, to CCR5 in pools of cervicovaginal secretions from HIV-seronegative healthy women (open squares) or HIV-seropositive women (bold squares) by isotype. The specific activities of antibodies of the IgA, IgG, or IgM isotype to the CCR5 peptide in CVL fluid samples were evaluated at the first dilution that gave an OD between 0.5 and 1.5 and per µg of total IgA, IgG, and IgM antibodies in CVL fluid samples, respectively, according to the formula $A = (OD - d)/[Ig]$ (in µg/ml), as described previously (3, 40). The results are expressed as the means of the values obtained from three independent experiments ± SDs. * $P < 0.05$ by the paired Student $t$ test between specific activities of CVL fluid samples from HIV-seronegative and HIV-seropositive women.
peptide, suggesting differential avidities to CCR5 of CCR5-specific antibodies in CVL fluid samples. We further assessed the avidities of CCR5-specific CVL fluid antibodies by determining the abilities of increasing concentrations of KSCN to dissociate bound antibodies from the CCR5 peptide (6, 40). The molarities of KSCN required to dissociate 50% of total anti-CCR5 antibodies from the CCR5 peptide ranged from 0.25 to 0.75 M for antibodies purified from two HIV-seronegative women (CVL fluid samples 2 and 14) and two HIV-seropositive women (CVL fluid samples 24 and 28). No difference in the relative avidity was observed between the antibodies purified from CVL fluid samples from HIV-seronegative and HIV-seropositive women (Fig. 4C).

Natural anti-CCR5 antibodies from cervicovaginal secretions recognize and bind to CCR5 expressed on HIV target cells. We further characterized the ability of CCR5-reacting NAbs, isolated from HIV-seronegative CVL fluid samples, to interact with physiological CCR5-expressing cells such as MDMs, immature MDDCs, and T lymphocytes. In total, 91% ± 4.3%, 45% ± 2.1%, and 10% ± 1.3% of MDMs, immature MDDCs, and T lymphocytes, respectively, expressed CCR5, as evaluated by CCR5-specific MAb binding (Fig. 5A). As depicted in Fig. 5B, affinity-purified anti-CCR5 antibodies from cervicovaginal secretion pools bound in a dose-dependent manner to the surfaces of MDMs and immature MDDCs. A plateau of binding was reached at a CCR5-reactive antibody concentration of 1,000 µg/ml, which stained 75% ± 6.9% and 45% ± 2.0% of MDMs and immature MDDCs, respectively. Lymphocytes bound to only 3% ± 0.6% and 7% ± 0.2% of NAbs when they were used at 100 and 1,000 µg/ml, respectively. Less than 5% binding was observed when anti-CCR5-depleted NAbs were used (Fig. 5B).

We further analyzed the specificities of CCR5-reacting NAbs toward CCR5 expressed on MDMs and MDDCs. Cells were preincubated with anti-CCR5 NAbs and were subsequently incubated with mouse anti-human CCR5 MAbs and goat anti-human Ig antibodies in order to characterize the competition between anti-CCR5 MAbs and NAbs. As depicted in Fig. 5C, anti-CCR5 MAbs bound very efficiently on MDMs and MDDCs without preincubation of the anti-CCR5 NAbs. However, in the presence of preincubation of anti-CCR5 NAbs, a faint binding of anti-CCR5 MAbs was detected. Taken together, these data suggest that anti-CCR5 NAbs can interact specifically with CCR5 physiologically expressed on MDMs and MDDCs.

Natural anti-CCR5 antibodies from cervicovaginal secretions inhibit CCR5-dependent HIV infection of MDMs and MDDCs. MDMs or MDDCs were infected with HIV BaL or HIVJR-CSF in the presence or absence of affinity-purified anti-CCR5 NAbs. The supernatants were harvested at 6 days postinfection, and the HIV p24 antigen concentrations were measured by ELISA. As depicted in Fig. 6, affinity-purified anti-CCR5 antibodies from pooled CVL fluid samples inhibited infection of both MDMs and immature MDDCs with HIVBaL and HIVJR-CSF in a dose-dependent manner. Thus, at concentrations of 500 and 1,000 µg/ml, the anti-CCR5 antibodies significantly inhibited the infection of MDMs with HIVBaL by 67% and 81%, respectively (690 ± 280 and 400 ± 164 pg/ml of HIV p24, respectively, in the presence of antibodies versus 2,150 ± 156 pg/ml of HIV p24 in the absence of antibodies) (Fig. 6A). As expected, MAb 2D7, which specifically blocks the CCR5-dependent entry of HIV-1, achieved 90% inhibition (240 ± 150 pg/ml of HIV p24 in the presence of antibodies versus 2,150 ± 156 pg/ml in the absence of antibodies). Less than 10% inhibition was obtained in the presence of depleted CCR5 NAbs in CVL fluid samples. Under the same conditions, the infection of MDMs with primary isolate...
HIVJR-CSF was significantly inhibited by 48% and 75% (490 ± 80 and 230 ± 64 pg/ml, respectively, in the presence of antibodies versus 950 ± 156 pg/ml in the absence of antibodies), as illustrated in Fig. 6C.

The infection of immature MDDCs by HIVdL (Fig. 6B) and HIVJR-CSF (Fig. 6D) was significantly but modestly inhibited by CCR5-reactive NAbs by 38% (312 ± 45 pg/ml in the presence of antibodies versus 649 ± 60 pg/ml in the absence of antibodies)
FIG. 5. Binding of anti-CCR5 NAbs to macrophages (CD14⁺), immature dendritic cells (CD1a⁺), and T lymphocytes (CD3⁺) at the single-cell level. (A) MDMs, MDDCs, and T lymphocytes (10⁵) were incubated with anti-CD14-FITC, anti-CD1a-FITC, and anti-CD3-FITC MAbs, respectively, and with an anti-CCR5 PE MAb (2D5 clone; 10 μg/ml) at 4°C for 30 min. (B) MDMs, MDDCs, and T lymphocytes (10⁵) were incubated with mouse anti-CD14 PE, anti-CD1a PE, and anti-CD3-PE MAbs, respectively, and with either 0, 100, or 1,000 μg/ml of purified anti-CCR5 NAbs or 1,000 μg/ml of anti-CCR5-depleted NAbs at 4°C for 30 min. (C) MDMs and MDDCs (10⁵) were incubated with or without 1,000 μg/ml of purified anti-CCR5 NAbs at 4°C for 30 min. The cells were subsequently incubated with FITC-conjugated goat anti-human F(ab')₂ antibody and an anti-CCR5 PE MAb at 4°C for 30 min. The percentage of positive cells is indicated in quadrants defined according to the relevant isotypic control. The results of one representative experiment of four independent experiments conducted are shown.
FIG. 6. Inhibition of infection of macrophages and dendritic cells with R5-HIV_{Ba-L} and R5-HIV_{JR-CSF} by anti-CCR5 NAb purified from CVL fluid samples from HIV-seronegative women. MDMs (A, C, and E) and MDDCs (B, D, and F) were preincubated with either anti-CCR5 affinity-purified NAb (50, 500, and 1,000 µg/ml), anti-CCR5-depleted NAb (1,000 µg/ml; negative control), anti-CCR5 MAb 2D7 (10 µg/ml), or an anti-CXCR4 MAb (10 µg/ml) prior to infection with R5-HIV_{Ba-L} (A and B), R5-HIV_{JR-CSF} (C and D), or X4-HIV_{NDK} (1 ng/ml) (E and F) at 37°C for 3 h. The level of production of the HIV p24 antigen in the culture supernatants was determined at day 6 postinfection by ELISA. The results are expressed as the mean HIV p24 concentrations from three independent experiments ± SDs. *, P ≤ 0.05 by the paired Student t test compared to the HIV p24 concentration in medium; **, P ≤ 0.01 by the paired Student t test compared to the HIV p24 concentration in medium.
antibodies) and 43% (312 ± 40 in the presence of antibodies versus 549 ± 65 pg/ml in the absence of antibodies), respectively, when the NAbs were used at a high concentration (1,000 μg/ml). Anti-CCR5 MAb 2D7 blocked the infection of immature MDDCs with HIV BaL and HIVJR-CSF by 66% and 78%, respectively. No inhibition was observed when the anti-CCR5-depleted fraction was used at a high concentration (1,000 μg/ml).

The infection of MDMs and MDDCs with primary HIV strain NDK, a CXCR4-tropic strain, was used as a control (Fig. 6E and F). No significant inhibition of either MDM and MDDC infection was observed when affinity-purified anti-CCR5 NAbs or the anti-CCR5 MAb was used. Conversely, anti-CXCR4 MAb, which was used as a positive control, blocked the infection of MDMs and immature MDDCs by 55% and 22%, respectively (Fig. 6E and F).

No inhibition of HIV BaL or HIVJR-CSF infection of MDMs and MDDCs could be observed when 100, 500, and 1,000 μg/ml of the irrelevant myeloma IgG was used as a control.

Anti-CCR5 NAbs from cervicovaginal secretions partially inhibit CCR5-dependent HIV transfer from immature MDDCs to T lymphocytes. Finally, we characterized the ability of anti-CCR5 NAbs to block the transfer of HIV-1 from immature dendritic cells (immature MDCs) to autologous T lymphocytes. As shown in Fig. 7, only a high concentration (1,000 μg/ml) of anti-CCR5 NAbs achieved significant inhibition of the transfer of HIV BaL and HIV JR-CSF from immature MDDCs to lymphocytes (33% and 44% inhibition, respectively; 4,312 ± 450 and 6,490 ± 600 pg/ml, respectively, in the presence of antibodies and 2,512 ± 450 and 4,490 ± 800 pg/ml, respectively, in the absence of antibodies) (Fig. 7A and B). In addition, the transfer of HIV NDK was used as a control (Fig. 7C). As reported for HIV infection, no significant inhibition of
X4-HIV transfer from MDDCs to T cells was observed when anti-CCR5 NAbs or anti-CCR5 MAb were used. Conversely, anti-CXCR4 MAb, which were used as a positive control, significantly blocked the transfer of HIVNDK to lymphocytes (33%), whereas no inhibition was observed with anti-CCR5 MAb 2D7 (Fig. 7C).

**DISCUSSION**

In the present study, we demonstrated that human cervicovaginal secretions contain NAbs (e.g., polyreactive antibodies). In addition, we found that NAbs directed against CCR5 were present in female genital secretions, were able to inhibit the infection of human macrophages and dendritic cells with primary R5-HIV, and limited the transfer of HIV from dendritic cells to T cells in vitro.

Our study demonstrated that the female genital tract contains large amounts of Igs that are mostly of the IgG isotype rather than the IgA isotype, which is in contrast to the findings for other mucosal secretions, such as breast milk, tears, and saliva, in which IgA is mostly predominant (4, 13, 23, 44). IgM antibodies were only faintly detected. If IgA antibodies were locally produced, as suggested by the presence of the secretory component in all samples, IgM antibodies are mostly thought to come from the systemic compartment, like a proportion of IgG antibodies, which may thus exhibit dual origins: (i) local production and (ii) transudation from the plasma (2). However, as CVL fluid contains material originating from the cervix and the vagina, determination of the precise location of both processes in not possible by this collection method. Interestingly, the pattern is very close to that observed in seminal plasma (36). Our study also demonstrated that CVL fluid samples from seropositive women exhibited a dramatic increase in IgG and IgM contents. This increase may be related to a stronger transudation process from the plasma in the female genital tract during HIV infection, as previously described by our group (5, 7). Regarding specific activity, our results indicate that NAbs are of the IgG, IgA, and IgG isotypes, whether they are from HIV-seronegative or HIV-seropositive donors. If strong natural specific activities of IgA and IgM were expected in a mucosal context (44), the strong natural activity of IgG is also in agreement with a dual source of IgG from the mucosal and plasmatic compartments. In addition, our observations demonstrate that the levels of NAbs in cervicovaginal secretions are increased in HIV-infected women (compared to those in HIV-negative controls), as previously demonstrated for cervicovaginal polyclonal antibodies of unknown specificities (23).

Natural reactive autoantibodies are thought to be involved in immune homeostasis (25, 32). Therefore, the existence of CCR5-reactive antibodies could limit the migration of CCR5+ proinflammatory cells, such as macrophages, dendritic cells, CD8+ effector T cells, and Th1 cells, toward inflammatory sites producing RANTES, MIP-1α, or MIP-1β. We also report on the presence of CCR5-specific natural IgM antibodies in CVL fluid samples. Interestingly, even if these antibodies were present at low concentrations, they would exhibit strong specific activities toward autoantigens and especially toward CCR5. As cervicovaginal IgM antibodies were thought to originate from the plasmatic compartment, it would be interesting to characterize natural plasmatic CCR5-specific IgM antibodies. Physiologically, these CCR5-reactive IgM antibodies may also participate in immune homeostasis by deleting circulating CCR5+ proinflammatory cells in a complement-dependent manner, as previously reported for natural xenoreactive IgM antibodies (38). Similarly, CCR5-reactive IgG antibodies may also induce the cytotoxicity of CCR5+ cells in an antibody-dependent cell-mediated cytotoxic manner (38). However, due to the small amounts of material available, we could not confirm these hypotheses.

HIV infection is predominantly transmitted through mucosae (8, 35). After it crosses the epithelial barrier, HIV spreads rapidly through contact between dendritic cells and CD4+ T lymphocytes (35). The CCR5 chemokine receptor functions as the major coreceptor for strains that use CCR5, which are primarily transmitted through the mucosal route (30). The role of CCR5 in HIV-1 transmission has been evidenced by observations that individuals homozygous for a defective CCR5 allele (CCR5 Δ32) remained uninfected, despite repeated exposure to HIV (24, 34, 41, 48), and that protection against HIV infection was associated with the presence of natural anti-CCR5 antibodies in serum in some exposed uninfected individuals in serologically discordant couples (31). The presence of anti-CCR5 antibodies has previously been documented in the sera of HIV-seropositive individuals (21), HIV-exposed uninfected individuals (31), healthy individuals (12, 21), and homozygous CCR5 Δ32 individuals who had been repeatedly exposed to CCR5-expressing cells through sexual activity (18). Anti-CCR5 antibodies were shown to downmodulate the surface expression of CCR5 and to neutralize the infectivity of R5-HIV strains, providing a basis for the acquisition of resistance to infection (31). Hence, the blockade of CCR5 with mucosal anti-CCR5 antibodies could result in inhibition of the initial spread of viruses that use CCR5.

We also examined the effects of anti-CCR5 antibodies purified from CVL fluid samples on the infection of macrophages and dendritic cells that express CCR5 and are considered the primary targets of HIV after the virus crosses the epithelial barrier (10, 27, 52).

We demonstrate here that natural anti-CCR5 antibodies from CVL fluid samples from HIV-seronegative women, similar to anti-CCR5 MAb 2D7, dramatically reduce the levels of infection of macrophages and dendritic cells with R5-HIVNDK and R5-HIVJR-CSF at doses rather greater than 1,000 μg/ml. The inhibition of MDM and immature MDDC infection and that of immature MDDC infection from autologous T lymphocytes by anti-CCR5 antibodies were obtained in vitro by anti-CCR5 antibody concentrations within the physiological ranges observed in vivo in cervicovaginal secretions. Thus, considering that the CVL process induces a 10-fold dilution of the concentration of bioactive molecules present in CVL fluid samples (6) and that concentrations of natural anti-CCR5 antibodies in CVL fluid samples greater than 100 μg/ml (and, thus, greater than 1,000 μg/ml in CVL fluid samples) were found in almost all CVL fluid samples, we can suggest that in vivo these antibodies may exert a physiological role in the natural relative protection against HIV infection of CCR5+ target cells in the female genital tract. However, six CVL fluid samples exhibited low levels of anti-CCR5 NAbs. As previously described for the local secretion of plasmatic NAbs, interindividual variations of...
the NAbS in the genital tract can also exist, especially for IgA and IgG and the local expression of CCR5 under the regulation of hormonal control within the female genital tract (28, 33, 39).

In conclusion, the data presented here emphasize the role of CCR5 as a capital target in the inhibition of HIV mucosal transmission within the female genital tract, as suggested by recent developments with anti-HIV microbicides (26, 29, 45, 46, 50). Our study also emphasizes the influence of natural innate immunity in the early control of HIV genital transmission and, in particular, the role of NAbS, as reported in other studies (11, 42). Taken together, complementary data from our work and from a recently published study (39) strongly suggest that anti-CCR5 NAbS may be involved in the innate immune network controlling the genital transmission of HIV and should be considered during the development of anti-CCR5 microbicides.

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