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Opsonization of HIV-1 by Semen Complement Enhances Infection of Human Epithelial Cells

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In the present study we demonstrate that both X4- and R5-tropic HIV-1 strains are able to infect the human epithelial cell line HT-29. Infection was enhanced 2-fold when HIV was added to semen before contact with the cell cultures. The enhancing effect of semen was complement dependent, as evidenced by blockage of generation of C3a/C3a\textsubscript{desArg} in semen by heat or EDTA treatment of semen and suppression of semen-dependent enhancement with mAbs directed to complement receptor type 3 (CD11b/CD18) and soluble CD16. Infection of HT-29 cells was assessed by the release of p24 Ag in cultures and semiquantitative PCR of the HIV-1 pol gene. Inhibition of infection of HT-29 by stromal cell-derived factor 1 was decreased in the case of semen-opsonized X4- and R5-tropic virus compared with unopsonized virus. In contrast, inhibition of infection by RANTES was increased for opsonized X4-tropic HIV-1 compared with unopsonized virus. Taken together these observations indicate that activation of complement in semen may play an enhancing role in mucosal transmission of HIV-1 by facilitating infection of epithelial cells and/or enhancing infection of complement receptor-expressing target cells in the mucosa. *The Journal of Immunology*, 2002, 169: 3301–3306.

The complement system plays an important role in innate host defense mechanisms such as immune bacteriolysis, neutralization of viruses, immunocoagglutination, and enhancement of phagocytosis. The role of complement components present in biological fluids in mucosal HIV-1 transmission has been neglected. Transmission of HIV-1 may occur following the passage of virus through monostratified mucosal epithelium, such as the endometrial cell monolayer of the endocervix upon sexual transmission of HIV-1 and the intestinal mucosa upon postnatal transmission of virus by breast milk (1). Semen, cervicovaginal secretions, and breast milk of HIV-1-seropositive individuals contain cell-free HIV-1 particles (2, 3) and soluble complement components (4–9). Opsonization of HIV-1 with complement was shown to result in enhanced viral infection of T and B cell lines (10–12), primary PBMC (13), and primary monocyte/macrophage cultures (14). Since CD11b/CD18 (complement receptor type 3 (CR3)) is expressed on the apical surface of epithelial cells as well as on dendritic cells and macrophages in the submucosa, it may be speculated that opsonization of HIV with complement is important in the early events surrounding mucosal transmission of HIV (15, 16). In the present study we demonstrate that HIV in semen may infect human epithelial cells in a complement-dependent fashion.

**Materials and Methods**

**Reagents and Abs**

Seminal fluid from 10 HIV-1-seronegative healthy donors was obtained from the semen bank of Hôpital Tenon (Paris, France). The semen was centrifuged for 10 min at 4°C, and the supernatant was stored at −70°C. Anti-CD4 mAb (FITC-CD4, Leu3a) was obtained from BD Biosciences (Mountain View, CA); anti-CCR5 (PE-CCR5, 2D7) and anti-CXCR4 (PE-CXCR4, 12G5) mAbs were obtained from BD Pharmingen (Le Pont de Claix, France). PE-conjugated mAbs against CD11b/CD18 (CR3) and CD11c/CD18 (CR4) and unlabelled anti-CD11b/CD18 mAb MO-1/7E4 were purchased from Immunotech (Beckman Coulter, Villepinte, France). Recombinant RANTES and stromal cell-derived factor 1 (SDF-1) were obtained from R&D Systems Europe (Abingdon, U.K.). Soluble CD16 (sCD16) was a gift from C. Sautes (Institut Curie, Paris, France).

**Human epithelial cell line and sources of HIV-1**

HT-29 human colorectal epithelial cells line (ATCC HTB-38; American Type Culture Collection, Manassas, VA) was maintained in RPMI 1640 containing 10% FCS and antibiotics. Primary R5-tropic HIV-1BaL and HIV-1 JRCSF were amplified in primary HIV-1VN-44 and HIV-1 NDK X4-tropic viruses (gifts from Prof. F. Barre-Sinoussi, Institut Pasteur, Paris, France) were grown in PBL of healthy donors stimulated with PHA and IL-2. Primary R5-tropic HIV-1\textsubscript{DKD} and HIV-1\textsubscript{MCDF} were amplified in monocyte-derived macrophages of healthy donors. Tropism of viruses was determined using U87 cells (provided by Dr. E. Menue, Institut Pasteur) transfected with DNA encoding for human CD4, CCR5, or CXCR4. HIV was quantified in cell culture supernatants by means of the DuPont HIV-p24 ELISA (HIV-1 core profile ELISA; DuPont de Nemours, Les Ulis, France).

**Opsonization of HIV-1 and in vitro infection of HT-29 epithelial cells**

Seminal fluid and normal human serum obtained from HIV-seronegative individuals were used as sources of complement. We estimated levels of C3 fragments in semen to be ~10% of those in serum. C3 and C9 complement fragments levels in semen have been previously reported to be between 0.3 and 5% of those in blood plasma (S. E. Bozaj, unpublished observations). HIV-1 particles were opsonized by adding seminal fluid or normal human serum diluted in veronal buffer supplemented with 0.6 mM CaCl\textsubscript{2} and 0.9 mM MgCl\textsubscript{2} to HIV-1 (10 ng p24) for 1 h at 37°C (opsonized virus). As negative control for opsonized virus, HIV-1 particles were incubated with heat-inactivated seminal fluid (HI-SF) or normal human serum (NSS).
heat-inactivated serum (HI unopsonized virus). For infection of epithelial cells, 10^5 HT-29 cells were inoculated with opsonized X4-tropic (HIV-1_VN-44, HIV-1_HRD) or R5-tropic (HIV-1_Bal, HIV-1_JRCSF) strains (10 ng p24) or with similar amounts of the corresponding unopsonized viral particles for 3 h at 37°C. The cells were then washed with RPMI 1640, resuspended in fresh RPMI 1640 containing FCS, and cultured for 3 days. Cell culture supernatants were collected at 24-h intervals, and viral content was monitored by HIV-1 p24 ELISA.

**Membrane expression of CRs and HIV-1 coreceptors by HT-29 cells**

The expression levels of CCR5, CXCR4, CR3, and CR4 by HT-29 epithelial cells were investigated by flow cytometry. The cells were detached by adding veronal (1/1000; Life Technologies, Cergy Pontoise, France) to culture plates for 10 min at 37°C, washed with PBS containing sodium azide (0.01%) and BSA (0.2%), and then incubated with PE-conjugated anti-CD4, anti-CCR5, anti-CXCR4, and anti-CR3/CR4 mAbs or with unlabeled isotype-matched mAbs for 30 min at 4°C. After washing, the cells were fixed with paraformaldehyde (1%) and analyzed using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences).

**Detection of HIV-1 DNA in HT-29 cells**

Levels of HIV-1 DNA in HT-29 cells were investigated 3 h after infection with opsonized or unopsonized virus. DNA was extracted from washed cellular pellets using the QIAamp DNA mini kit (Qiagen, Basel, Switzerland) according to the manufacturer’s instructions. A semiquantitative PCR of the pol gene was then performed as previously described (17). PCR was performed with the oligonucleotides P63 (5'-GCC ATT TAA AAA TCT GAA AAC AGG-3') and P58 (5'-GAC AAA CTC CCA CTC AGG AAT CCA-3') for 37 cycles (94°C for 30 s, 53°C for 30 s, and 72°C for 60 s) in a reaction volume of 50 μl containing Milli-Q water, DNA templates, reaction buffer provided by the manufacturer, 1.5 mM MgCl2, 200 μM of each dNTP, 250 nM of each primer, and 2.5 U Taq DNA polymerase (Promega France, Charbonnières, France). To check for the quality of extracted DNA and the lack of PCR inhibitors, the ubiquitous β-globin gene was amplified in parallel experiments by PCR as previously described (18). The final PCR products were visualized under UV transillumination by means of ethidium bromide staining after electrophoresis in 2% agarose.

**Results**

**Complement in seminal fluid is activated by R5- and X4-tropic strains of HIV-1**

To investigate the ability of HIV to activate complement in seminal fluid, HIV-1 particles (10 ng/ml p24) were added to seminal fluid diluted in VBS+ (v/v; final volume, 100 μl) and incubated for 1 h at 37°C. After centrifugation, the generated C3a Ag was then determined in supernatants by ELISA (OPT EIA Human C3a ELISA; BD PharMingen, San Diego, CA). HI-SF and 20 mM EDTA-chelated seminal fluid were used as negative controls. Complement activation by heat-aggregated IgGs (1 mg/ml) in seminal fluid was used as a positive control. As shown in Fig. 1, incubation of R5- or X4-tropic strains of HIV-1 particles with seminal fluid resulted in activation of complement. X4-tropic (HIV-1_VN-44) and R5-tropic (HIV-1_Bal) strains yielded similar levels of complement activation, reaching values comparable to those obtained with heat-aggregated Ig. Only low levels of C3a/C3adesArg Ag were detected when HIV-1 or heat-aggregated Ig was incubated with heat-inactivated or EDTA-chelated seminal fluid.

**CRs and HIV-1 coreceptors are expressed by HT-29 cells**

Ninety-four percent of HT-29 cells expressed a high level of CXCR4 (mean fluorescence intensity (MFI), 154); 35% of the cells were positive for CCR5 (MFI, 30–40), whereas very low (0.1%) expression of CD4 Ag was detected (Fig. 2a). We further observed that 45% of HT-29 epithelial cells expressed CR3 (CD11b/CD18), and 35% expressed CR4 (CD11c/CD18; MFI, 20 and 15, respectively; Fig. 2b). There was no expression of CR1 (CD35) or CR2 (CD21; data not shown). Coexpression levels of HIV-1 coreceptors and CR3 on HT-29 cells were further established by double-staining fluorescence with CCR5 (2D7)/CR3(MO-1) mAbs and CXCR4(12G5)/CR3 mAbs to be 22 and 35%, respectively (not shown).

**Human HT-29 epithelial cells are susceptible to infection with primary R5- and X4-tropic strains of HIV-1**

We found that both X4-tropic (HIV-1_NDK and HIV-1_VN-44) and R5-tropic (HIV-1_Bal and HIV-1_JRCSF) strains of HIV were able to infect HT-29 cells (Fig. 3). A plateau of p24 Ag production was reached 72 h postinfection. At the peak of infection, the levels of HIV-1 p24 Ag released after in vitro infection with HIV-1_NDK (1000 pg/ml) and HIV-1_VN-44 (900 pg/ml) were higher than those achieved with R5-tropic HIV-1_Bal (350 pg/ml) and HIV-1_JRCSF (250 pg/ml).

**Complement-dependent enhancement of HIV-1 infection of HT-29 cells**

Infection of HT-29 cells with HIV-1_Bal and HIV-1_VN-44 (Fig. 4) particles that had been pre-opsonized with complement in seminal fluid resulted in an enhanced production of HIV-1 p24 Ag in culture cell supernatants compared with that observed upon infection with the corresponding unopsonized virus. The enhancement achieved at 72 h of culture ranged between 1.5- and 2-fold. No enhancement of infection was observed when viruses were incubated with HI-SF. Semiquantitative PCR was then used to quantify HIV-1 DNA in HT-29 cells following infection with opsonized or
unopsonized virus. Viral DNA was measured after 3 h of ongoing infection with HIV-1 BaL and HIV-1 VN-44 (Fig. 5). A greater amount of DNA was present in cells infected with opsonized virus than in those infected with unopsonized virus. When complement was heat inactivated in semen before opsonization, viral DNA levels measured in the cells were comparable to those detected in the unopsonized virus. Infection experiments of HT-29 cells by HIVNDK and HIV JRCSF strains, with or without recombinant soluble CD4 molecule (Intracel, Issaquah, WA), showed no inhibition of HT-29 cell infection in the presence of soluble rCD4 molecule, thus demonstrating that the CD4 receptor is not involved in viral entry into HT-29 epithelial cells (data not shown).

Role of chemokine receptors in infection of HT-29 cells by complement-opsonized HIV-1

To investigate the role of chemokine receptors expressed by HT-29 cells for viral entry, cells were incubated with RANTES or SDF-1 (2.5 μg) before the addition of virus. SDF-1 inhibited 90% infection of HT-29 cells with X4-tropic HIV-1NDK and 85% infection with HIV-1VN-44 (Fig. 6) in a dose-dependent manner (data not shown). No significant inhibition of infection was obtained by RANTES (<10%), with X4-tropic HIV-1NDK and HIV-1VN-44. Surprisingly, only 20 and 30% inhibitions were reached for HIV-1BaL and HIV-1JRCSF, respectively, in the presence of rCD4 molecule, thus demonstrating that the CD4 receptor is not involved in viral entry into HT-29 epithelial cells (data not shown).

CR3 dependency of infection of HT-29 by complement-opsonized HIV-1 in seminal fluid

To investigate the role of CR3 receptor in the infection of HT-29 epithelial cells, cells were incubated with anti-CR3 mAbs and soluble sCD16, one of the natural ligands of CR3, before infection with complement-opsonized HIV-1BaL and HIV-1VN-44. HT-29 cells (10^5 cells) were incubated with anti-CR3 mAbs (MO-1/7E4; 10 μg), human recombinant sCD16 (5 μg), and IgG1 isotype as a negative control for 30 min before infection with opsonized or unopsonized HIV-1VN-44 and HIV-1BaL. Cells were washed and further cultured for 3 days. Cell supernatants were collected every 24 h. As shown in Fig. 7, the addition of either sCD16 or a mixture of anti-CD11b and anti-CD18 mAbs abolished the enhancing effect of complement opsonization at 72 h postinfection. Upon blockage of CR3 receptors, HIV-1 p24 Ag levels measured in culture supernatants were similar to those observed in the presence of HI-SF (data not shown).

Opsonized HIV-1 modulates the use of coreceptors by X4- and R5-tropic HIV strains

SDF-1 inhibited 71 ± 2 and 86 ± 8% of p24 production by HT-29 cells infected with HI-SF-opsonized HIV-1BaL and HIV-1VN-44, respectively. When cells were infected with SF-opsonized virus, the inhibition of p24 production observed upon preincubation of the cells with SDF-1 was significantly reduced (53 ± 1% of inhibition for HIV-1BaL and 54 ± 13% for HIV-1VN-44). The inhibitory effect of SDF-1 was significantly different when comparing p24 production in SDF-1-treated cells infected with HI-SF-opsonized or SF-opsonized virus (p = 0.01 and p = 0.008 for HIV-1BaL and HIV-1VN-44, respectively). In contrast to SDF-1, RANTES was more efficient in inhibiting infection of HT-29 cells with SF-opsonized HIV-1VN-44 (41 ± 19%) than with the corresponding HI-SF-opsonized virus (10 ± 5%; p = 0.008 for RANTES inhibitory effect on infection with HI-SF-opsonized vs SF-opsonized virus). No significant difference was observed with regard to
inhibition by RANTES of infection with HI-SF-opsonized (36 ± 15%) and SF-opsonized (42 ± 2%) HIV-1_Bal (Table I). As a control, the inhibitory potency of SDF-1 was assessed on infection of PBL with unopsonized HIV-1_VN-44 and that of RANTES using macrophages infected with unopsonized HIV-1_Bal. SDF-1 and RANTES inhibited infection by 80 and 95%, respectively (data not shown).

Discussion

In the present study we demonstrate that a human epithelial cell line is sensitive to infection with HIV in the presence of seminal fluid. Infection is a complement-dependent process and occurred with both R5- and X4-tropic primary isolates of HIV-1.

Upon culture in the presence of semen to which X4- or R5-tropic HIV-1 particles had been added, we observed a productive infection of HT-29 epithelial cells occurring from the first day of culture. The production of viral p24 protein in culture supernatants of infected HT-29 cells was increased up to 1.5- to 2-fold in the presence of a functional complement system in semen compared with infection observed in the absence of semen. Infection was also demonstrated by assessing the presence of proviral HIV-1 DNA in HT-29 cells following 3 h of incubation with HIV-1 in semen. The amounts of HIV-1 pol DNA measured by semiquantitative PCR were higher in cells infected with R5- or X4-tropic viruses that had been preincubated in semen compared with cells infected with virus in culture medium. This study provides evidence that complement in semen is involved in the enhancement of infection observed when HIV particles are preincubated with semen before their addition to the cell cultures. The role of complement in semen was strongly suggested by the observation that infection with HIV added to heated or EDTA-treated semen was no different from that seen with HIV alone (i.e., diluted in culture medium). Complement components have been detected in all body secretions, including seminal fluid (4, 5). HIV is known to activate complement in serum in the presence or the absence of Abs (12, 19). Here we show that both primary R5- and X4-tropic strains activate complement in the seminal fluid as assessed by the generation of the C3 cleavage fragments C3a/C3a(desArg). No C3 cleavage was observed when heat-inactivated and EDTA-chelated seminal fluid was used. Several regions in viral gp120/gp41, such as the conserved C2 region, the carboxyl-terminal flank of the V3 loop, and the transmembrane gp41 domain, have been shown to be involved in complement activation through direct interaction with C3 and/or C1q (20–22). Activation of complement results in deposition of C3 fragments on the viral surface. HIV-1 particles are, however, resistant to lysis by the complement membrane attack complex C5b-9 (23–25).

Since the seminal fluid of infected men contains both free HIV-1 particles (2, 3) and complement components (4), it may be speculated that virus in semen becomes opsonized and acquires an increased potential to infect epithelial cells or other target cells in the submucosa, e.g., dendritic cells, resulting in mucosal transmission of the virus. Mucosal transmission of HIV-1 through infection of epithelial cells remains a controversial issue (26–29). In the present study we report that HT-29 epithelial cells can be infected by R5- and X4-tropic strains of HIV-1. Our result differs from previous reports indicating that HT-29 cells can only be infected by X4-tropic HIV-1 (30).

FIGURE 4. Infection of epithelial cells HT-29 with complement-opsonized virus. Virus opsonization was conducted by incubation of both HIV-1_Bal and HIV-1_VN-44 with seminal fluid in VBS°+ for 1 h at 37°C before infecting cells. Cells were then washed, and p24 release was estimated by ELISA at 72 h postinfection. Results are expressed as the mean p24 production ± SD in three independent experiments.

FIGURE 5. HIV-1 DNA detection in HT-29 cells infected with seminal fluid complement-opsonized (SF-OV) and unopsonized (UV) HIV-1_Bal and HIV-1_VN-44. Total DNA was extracted from 3-h infected cells, and viral DNA was detected by PCR of the HIV-1 pol gene. The negative control was heat-inactivated semen complement (HI-SF-OV). PCR specificity was checked using uninfected HT-29 cells (T). Constant amounts of extracted DNA were used, as shown by amplification of the ubiquitous β-globin gene.
is thought occur during sexual intercourse, especially during peno-anal intercourse. To investigate the receptors involved in viral entry in HT-29 epithelial cells, we first analyzed the membrane expression of CD4, CXCR4, CCR5, CR3 (CD11b/CD18), and CR4 (CD11c/CD18) by HT-29 cells. Most cells expressed CXCR4 (94%), CCR5 (35%), and CR3 (45%). HT-29 cells did not express the CD4 molecule. Our results obtained with the epithelial cell line HT-29 are consistent with previous observations of the expression of CXCR4, CCR5, and CR3 (CD11b/CD18) by mucosal epithelial cells (31–33). These receptors are also known to be expressed by dendritic cells and macrophages, which have been suggested to be the first targets for HIV-1 in the submucosa upon sexual transmission of the virus (15, 16, 34). Infection of HT-29 cells with HIV-1NDK and HIV-1VN-44 X4-tropic viruses that had not been preopsonized with semen was totally inhibited by SDF-1, the natural ligand of CXCR4. Interestingly, SDF-1 also inhibited infection when HT-29 cells were infected with HIV-1NDK and HIV-1VN-44, two R5-tropic strains. In contrast, RANTES, the natural ligand of the CCR5 receptor, was a poor inhibitor of infection with R5-tropic viruses (HIV-1JRCSF and HIV-1BaL) and, as expected, had no effect on infection with X4-tropic viruses (HIV-1NDK and HIV-1VN-44). Taken together, our results indicate that unopsonized X4- and R5-tropic viruses use CXCR4 as a coreceptor to infect CD4-negative epithelial cells. Infection of HT-29 cells could also implicate additional receptors, such as galactosylceramide (Galler), that are expressed on HT-29 cells (31, 35, 36). Abs to Galcer induced 40–50% inhibition of infection with R5- and X4-tropic unopsonized HIV-1, suggesting the participation of this receptor (data not shown).

Infection with virus that had been opsonized with complement in semen was inhibited by mAbs directed against the region of CR3 (CD11b/CD18) that recognizes the iC3b binding site and by sCD16, a natural ligand of the CR3 receptor. These results demonstrate that the enhancing effect of opsonization by semen was dependent on CR3-mediated entry of opsonized virus in HT-29 cells. These data extend our previous observations on the enhancing effect of complement opsonization in serum on infection of monocytes/macrophages (14). We further observed that inhibition of infection by SDF-1 was decreased in the case of infection with opsonized virus compared with that observed with unopsonized R5- and X4-tropic viruses. In contrast, inhibition induced by RANTES was higher for opsonized X4-tropic HIV-1VN-44 than for the corresponding unopsonized virus. These data suggest that opsonized virus does not interact with CCR5 and CXCR4 on epithelial cells in the same way as the corresponding free unopsonized virus. It is possible that the presence of iC3b on viral particles affects the interactions of HIV with CCR5 and CXCR4 on target cells. HIV-1 would thus turn complement opsonization to its own replicative advantage, resulting in enhanced infection of epithelial cells and possibly of other cell types in the submucosa, thereby promoting the spread of the virus.

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