Male reproduction and HIV-1 infection
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Human immunodeficiency virus-type 1 susceptible cells in semen of HIV-1-positive and HIV-negative men

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Submitted
Abstract

Background: In an HIV-1 sanctuary site viral replication continues during highly active antiretroviral therapy (HAART). A prerequisite for a sanctuary site is the absence of adequate local drug concentrations and the presence of cells susceptible to HIV-1. In the male genital tract (MGT), claimed to be a sanctuary site, concentrations of several antiretroviral drugs are indeed low, but whether HIV-1 susceptible cells are present is currently unknown.

Objective: To determine whether HIV-1-susceptible cells are present in semen of HIV-1-infected men using HAART, HIV-1-infected therapy-naïve men and HIV-negative men, and to compare HIV-1-susceptible cells in semen with HIV-1-susceptible cells in blood.

Methods: Seminal lymphocytes and blood lymphocytes were stained with monoclonal antibodies against CD45, HLA-DR and CD38, and analyzed using flow cytometry. CD45+HLA-DR+CD38+ cells were considered to be susceptible to HIV-1 infection.

Results: CD45+HLA-DR+CD38+ cells were detected in semen of all men, but the absolute number of these cells was extremely low. The percentage of CD45+ cells in semen that were HLA-DR+CD38+ was 14%, 16% and 11% for HIV-1 positive men on HAART, untreated HIV-1 positive men and HIV-negative men, respectively (P=.48, ANOVA). The corresponding percentages of CD45+HLA-DR+CD38+ cells in blood were 17%, 25% and 10%, respectively (P<.0001, ANOVA).

Conclusions: Semen contains cells susceptible to HIV-1. Whether these cells are present at sites in the MGT that are exposed to inadequate drug levels and whether the number of these cells is sufficiently high to be clinically relevant with respect to the selection or development of drug-resistant strains remains to be determined.
**Introduction**

A sanctuary site for human immunodeficiency virus-type 1 (HIV-1) is an anatomical site which is highly impermeable to –some– antiretroviral drugs, and in which viral replication continues during treatment. A sanctuary site thus allows local selection and/or development of drug-resistant strains potentially causing therapy failure 1-3. Sanctuary sites for HIV-1 are characterized by two phenomena: –1– the absence of adequate local drug concentrations and –2– the presence of susceptible and subsequently virus replicating and producing cells 4,5.

The male genital tract (MGT) has been suggested as a sanctuary site for HIV-1, because the non-nucleoside reverse transcriptase inhibitor (NNRTI) efavirenz and most protease inhibitors (PIs), both extensively used in current highly active antiretroviral therapy (HAART), do not penetrate well into seminal plasma 6-9. Whether or not the MGT is indeed a sanctuary site for HIV-1 is at present unknown, because data on the presence of HIV-1-susceptible cells in the MGT are lacking 10.

Here, we report an explorative study on the number of HIV-1-susceptible cells in semen of HIV-1-infected men using HAART, HIV-1-infected therapy naïve men and HIV-negative men in comparison to the number of these cells in blood of the same men.

**Methods**

**Patients**
Between September 2005 and October 2006 HIV-1 positive men using effective first-line HAART for at least 12 weeks (HAART group), and HIV-1 positive men who were antiretroviral therapy naïve (no HAART group) were recruited from the HIV outpatient clinic of the Academic Medical Centre (AMC), Amsterdam, the Netherlands. Patients were asked for symptoms of genitourinary infections and a ligase chain reaction (LCR; Abbott diagnostics, Illinois, U.S.A.) was performed on first void urine to exclude active *Chlamydia trachomatis* infection.

In addition, healthy men, voluntary donating semen in a donor program at the Centre for Reproductive Medicine of the AMC, were recruited for this study (HIV-negative group). All donors were proven negative for HIV-1/2, hepatitis B and C, HTLV and *Chlamydia trachomatis* infection. The study was approved by the Institutional Review Board of the AMC and all patients and donors gave written informed consent.
Preparation of samples

Semen was collected in a sterile container after masturbation. After liquefaction at 37°C, semen volume was measured. The number of round cells, which contain immature germ cells, leukocytes and other non-spermatozoal seminal cells was determined using a disposable counting chamber (Léjà products B.V., Nieuw Vennep, The Netherlands). The nature of the non-spermatozoal cells was determined on a semen smear by using the Diff Quick staining procedure (Dade Behring, Dudingen, Switzerland). Semen was diluted 1:1 with Hanks balanced salt solution (HBSS; Sigma St. Louis, MO, USA) and centrifuged for 10 minutes at 350g at room temperature. Subsequently, sperm was diluted with 0.6 ml HBSS.

On the day of semen collection four-and-a-half ml of blood was collected in an ethylenediamine-tetra-acetic acid (EDTA) tube to determine the number of HIV-1 susceptible cells in blood.

Immunofluorescence staining

Staining was performed with monoclonal antibodies (mAbs) as follows: 20 μl of CD45-PE-Cy5, 20 μl HLA-DR allophycocyanin (APC) and 20 μl CD38 R-phycoerythrin (R-PE) were added to aliquots of 200 μl sperm suspension and 100 μl of EDTA whole blood. As a control, CD45-PE-Cy5 alone was added to another 200 μl sperm suspension and 100 μl of EDTA whole blood. All mAbs were purchased from Becton Dickinson (BD biosciences, San Diego, USA). Following vortex mixing and 20 minutes incubation in the dark at room temperature, 2 ml of ammoniumchloride (8.3 g/l) was added to the triple stained and control samples. After 10 minutes PBS-plus, i.e. PBS supplemented with 10% pasteurized plasma solution (GPO; Sanquin, Amsterdam, the Netherlands) was added to these ammoniumchloride treated samples to wash the cells and the sample was centrifuged at 350g for 5 minutes. The supernatant was discarded and this wash step was repeated once. Finally, cell pellets were resuspended in 0.5 ml of PBS-plus with 1% paraformaldehyde, to fixate the cells, and stored at 4°C until flowcytometric analysis.

To establish gates for lymphocytes in the flowcytometric analysis, a semen sample was spiked with blood-derived lymphocytes stained with CD45 mAbs as described previously. Briefly, lymphocytes were extracted from EDTA blood, by adding an aliquot of 100 μl of EDTA blood to 2 ml ammoniumchloride (8.3 g/l) at room temperature. After incubation for 10 minutes HBSS was added and the sample was centrifuged for 10 minutes at 350g at room temperature. The supernatant was discarded and the pellet was resuspended in HBSS.
Fifty μl of the blood suspension was then used to spike 100 μl of unstained spermatozoa suspension. The mixed sample was then stained with CD45 mAbs as described above.

**Flow cytometry analysis**

All stained cell suspensions were analyzed with LSR II (Becton Dickinson, San Diego, USA) using CELL Quest and FACSDiva software. Data were displayed in a dot plot on the basis of the linear forward (FSC) and side scatter (SSC) properties of the cells, with lymphocytes having high forward scatter (FSC) and very low side scatter (SSC) characteristics. The semen sample spiked with blood lymphocytes was used to set the gates for tracking and, subsequently, the unspiked sample was analyzed.

**Statistical analysis**

The number of susceptible cells was expressed as the percentage of cells with lymphocyte characteristics based on FSC and SSC characteristics and expression of CD45, HLA-DR and CD38 (CD45+HLA-DR+CD38+). Inactive cells, considered not to be susceptible to HIV-1, expressed CD45 only (CD45+HLA-DR-CD38-). The median percentage of CD45+HLA-DR+CD38+ cells in semen and blood was calculated for all three study groups. Subsequently, a blood-to-semen-ratio of CD45+HLA-DR+CD38+ cells was calculated in all three study groups. A one-way-ANOVA test with post-hoc testing (Bonferroni) was performed to detect differences in the percentage of these cells in semen and blood between the three study groups. Statistical analyses were performed using SPSS v. 12.0.2 software (SPSS Inc., Chicago, IL, USA).

**Results**

Eight HIV-1-infected men using HAART (HAART group), eight HIV-1-infected therapy-naïve men (no HAART group), and eight healthy HIV-1/2-negative semen donors (HIV-negative group) were enrolled. All men were asymptomatic for genitourinary infections and none had *Chlamydia* infection.

Patient characteristics are described in Table 1. Median CD4 counts in the HAART and in the no HAART group were 330 (interquartile range [IQR] 225–430) and 390 (IQR 325–450) cells/μl, respectively. Median blood plasma HIV-1-RNA levels in the HAART and no HAART group were <50 (IQR<50–77) and 111,018 (IQR 7,824–135,210) copies/ml, respectively. Six out of eight men in the HAART group had blood plasma HIV-1-RNA levels below the lower limit of detection of 50 copies/ml, and all men using HAART had blood plasma HIV-1-RNA
levels below 100 copies/ml. Leukocytes, as determined by Diff Quick staining of a semen smear, were detected in three out of 24 semen samples. In FACS analysis, CD45+CD38+HLA-DR+ cells were found in all semen samples (Table 1). The exact amount of CD45+CD38+HLA-DR+ cells was impossible to determine through FACS analysis but was estimated not to exceed 10,000 cells.

Median percentages of CD45+ cells that were CD38+HLA-DR+ in semen and blood are displayed in Figure 1. Median percentages of CD45+ cells that were CD38+HLA-DR+ in semen in the HAART, no HAART and the HIV-negative group were 14% (IQR 10–21), 16% (IQR 12–21) and 11% (IQR 5–19), respectively. These differences were not statistically significant (P=.48, one-way ANOVA). Median percentages of CD45+ cells that were CD38+HLA-DR+ in blood in the HAART, no HAART and the HIV-negative group were 17% (IQR 11–20), 25% (IQR 20–27) and 10% (IQR 8–14), respectively. These differences were statistically significant (P<.0001,

<table>
<thead>
<tr>
<th>Table 1. Patients’ characteristics</th>
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<tr>
<td>Variable</td>
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<tr>
<td>Age (years)</td>
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<td>Years since 1st positive test</td>
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<td>Times gonorrhoea infection (n)</td>
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<td>CD8+ T cells (cells/μl)</td>
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<td>Total number of round cells (x10^6)</td>
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<td>Total number of leukocytes (x10^6)</td>
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<td>No. of samples that contained leukocytes based on Diff Quick® staining</td>
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<tr>
<td>No. of samples that contained CD45+CD38+HLA-DR+ cells in FACS analysis</td>
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Data are presented as medians with interquartile ranges (IQR), unless otherwise stated. ND = not determined, NA = not applicable

* as determined by Diff Quick® staining
one-way ANOVA). Post-hoc testing revealed statistically significant differences in the median percentages of CD45+ cells that were CD38+HLA-DR+ in blood between all groups: HAART versus no HAART: \( P = .003 \), HAART versus HIV-negative: \( P = .04 \), no HAART versus HIV-negative: \( P < .001 \) (Bonferroni).

**Figure 1.** Percentage of CD45+ cells expressing CD38 and HLA-DR in semen and blood. The difference between groups with respect to the percentage of CD45+ cells expressing CD38 and HLA-DR in semen was not significant (\( P = .48 \), one-way ANOVA), the difference between groups with respect to the percentage of CD45+CD38+HLA-DR+ cells in blood was highly significant (\( P < .0001 \), one-way ANOVA), and the difference between groups with respect to the ratio of CD45+CD38+HLA-DR+ cells in blood versus semen was not significant (\( P = .43 \), one-way ANOVA).
Although in most patients the percentage of CD45+ cells that were CD38+HLA-DR+ appeared to be lower in semen than in blood, this difference was not statistically significant.

The median ratio of the percentage of CD45+ cells that were CD38+HLA-DR+ in semen and the percentage of CD45+ cells that were CD38+HLA-DR+ in blood in the HAART, no HAART and the HIV-negative group were 0.8 (IQR 0.6–1.6), 0.7 (IQR 0.6–0.8) and 0.8 (IQR 0.5–2.6), respectively. Differences between groups were not statistically significant ($P=.43$, one-way ANOVA).

**Discussion**

In this explorative study, we showed that HIV-1-susceptible cells are present in semen of HIV-1-infected men using HAART, as well as in HIV-1-infected men without HAART and in HIV-negative controls, albeit in extremely low numbers. The percentage of CD45+ cells in semen that expressed both HLA-DR and CD38 was not significantly different between the groups studied. Although in most patients the percentage of CD45+ cells that were CD38+HLA-DR+ cells appeared to be lower in semen than in blood, this difference was not statistically significant.

Some methodological issues merit discussion. First, the use of CD45 mAbs and FACS forward scatter (FSC) and side scatter (SSC) properties to localize lymphocytes leaves some room for uncertainty. Ideally, one should use CD4 monoclonal antibodies, but pilot experiments showed that, in agreement with literature, these antibodies did not work well in semen (data not shown)\(^1\). Second, we only determined the presence of susceptible lymphocytes, i.e. CD45+ cells. We cannot rule out the presence of additional susceptible macrophages, which are CD45-, in semen. Third, we used ejaculated semen. Since biochemical changes in semen take place rapidly after ejaculation, we cannot exclude an effect of our semen processing methods on lymphocyte activation. Thus, our findings in the ejaculate may not fully reflect the situation of seminal fluid and cells within the MGT\(^1\). Further, the presence of susceptible cells in semen does not answer the question where the cells originated from; lymphocytes in ejaculated semen could very well be blood-derived and exposed to HAART before they are transported to the MGT. Subsequently, lymphocytes might not be present in parts of the MGT that are impermeable to antiretroviral drugs, like the testes. The best study design to evaluate the presence of HIV-1-susceptible cells in the male genital tract would be to perform biopsies of several parts of the MGT in healthy and in HIV-1-infected men, but such
studies are clearly not feasible. Finally, during local infections numbers and activation state of lymphocytes in semen may be significantly higher.

The localization of HIV-1-susceptible cells in the male genital tract is still unclear. A recent study reported the presence of HIV-1-replicating-CD68+ macrophages in the interstitium of cultured human testicular tissue after in-vitro infection with HIV-1, and another study in macaques found infected macrophages in testicular interstitial tissue of the epididymis and testis after the acute stage of simian immunodeficiency virus and simian/human immunodeficiency virus infection. However, the blood-testis barrier prevents migration of HIV-1-susceptible cells from the interstitium to the lumen of the male genital tract and therefore these cells are not ejaculated.

Only a few studies have provided convincing evidence for the presence of HIV-1-resistant strains in semen indicative of a sanctuary site in the MGT. Our finding of very low numbers of HIV-1-susceptible cells in semen could explain this low incidence of drug resistant strains in semen.

In conclusion, we demonstrated the presence of HIV-1-susceptible cells in semen of HIV-1-infected men using HAART, HIV-1-infected therapy-naïve men and HIV-1/2-negative men, albeit in extremely low numbers. The origin of these cells is still unclear. Whether these cells are present at sites in the MGT that are exposed to inadequate drug levels and whether the number of these cells is sufficiently high to be clinically relevant with respect to the selection or development of drug-resistant strains remains to be determined.

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