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Short Communication

HIV-1 Nucleic Acids Localize to the Spermatogonia and their Progeny

A Study by Polymerase Chain Reaction In Situ *Hybridization*

Gerard J. Nuovo,* Joanne Becker,* Aylin Simsir,* Michele Margiotta,* Ghada Khalife,† and Maria Shevchuk,†

From the Department of Pathology, SUNY at Stony Brook, Stony Brook, New York; and Department of Pathology,† Lenox Hill Hospital, New York, New York*

The purpose of this study was to determine the histological distribution of* in situ *polymerase chain reaction (PCR)-amplified HIV-1 nucleic acids in the male genital tract to elucidate the mechanism of sexual transmission of AIDS. Viral DNA was detected in the testicular tissue of 11 of 12 men with HIV-1 infection using the PCR* in situ *hybridization technique. The amplified viral DNA localized to many spermatogonia, spermatocytes, and rare spermatids. Relatively few viral infected macrophages were noted, mostly in the prostate. The viral infection was activated given the presence of cDNA sequences consistent with genomic and multiple spliced transcripts as determined by reverse transcription* in situ *PCR. PCR-amplified viral nucleic acids were not detected in the epithelia of the prostate, epididymus, seminal vesicles, or penis in men with AIDS nor in any genital tract tissues from three boys who died of AIDS acquired* in utero. *The demonstration that HIV-1 selectively infects the spermatogonia and their progeny suggests that this may serve as a primary source of venereal spread of the virus. Concomitant destruction of these cells by HIV-1 may also explain the marked inhibition of spermatogenesis and severe atrophy that characterizes the testes in AIDS. (Am J Pathol 1994, 144:1142–1148)

Sexual transmission of HIV-1 infection has become a major risk factor for the development of AIDS.[1] The specific cell types in the male genital tract that may be infected by HIV-1 and thus serve to transmit the disease are not well known. One study of semen from men with early stage AIDS noted that the virus was found by polymerase chain reaction (PCR) in the seminal fluid and inflammatory cells in a majority of cases but was not detected in the spermatozoa fraction.[2] However, another study based on PCR *in situ* hybridization stated that up to 1:100 spermatids in semen contains HIV-1 DNA (R. Pomerantz and O. Bagasra, personal communication). One immunohistochemical study reported that HIV-1 proteins could be found in rare germ cells in the testes, whereas another study refuted this, noting a detection rate of 39% in the testes with the viral proteins being present in lymphocytes and macrophages.[3,4]

Although AIDS can affect a variety of organ systems, the testes invariably will show marked histological changes.[4] Over 95% of testes from men with AIDS demonstrate atrophy that is characterized by retarded or absent spermatogenesis, interstitial fibrosis, and a predominance of Sertoli cells. There is a concomitant oligospermia and azospermia. The basis of these dramatic histological changes is not known, although it appears to be selective in the male genital tract because the prostate, seminal vesicles, and epididymus generally show minimal histological

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Address reprint requests to Dr. Gerard J. Nuovo, Department of Pathology, SUNY, Stony Brook, NY 11794-8691.

changes that when present are often limited to a nonspecific inflammatory infiltrate.[4]

The major impediment to the direct localization of HIV-1 proteins and nucleic acids is the relatively poor detection rates of the respective assays. Recently, several groups have reported the *in situ* detection of PCR-amplified HIV-1 DNA.[5–10] These studies have shown that the PCR *in situ* hybridization assay is more sensitive than either standard *in situ* analysis or immunohistochemistry, being able to detect one target copy per cell. Using this technique in conjunction with reverse transcription *in situ* PCR for detection of viral RNAs, one can determine whether infection is latent, in which the infected cell may contain either only one integrated copy of the viral DNA or low levels of nongenomic viral transcripts, or active where there is upregulation of genomic and multiple spliced viral transcripts.[7–9] *In situ* PCR studies for HIV-1 have shown that approximately 30% of CD4 cells are infected in the lymph nodes in the asymptomatic patient where the infection is mostly latent, that in advanced stage AIDS most CD4 cells are actively infected, and in the cervix the macrophage in the endocervical aspect of the transformation zone is the primary target of the virus.[5–10]

The purpose of this study was to determine the histological distribution of PCR-amplified HIV-1 DNA and cDNA in the male genital tract.

Materials and Methods

Case Selection

Genital tract tissues from 18 people were available for study. Eleven were men who had died of AIDS, 1 was a man who had asymptomatic HIV-1 infection, 3 were children less than 3 years old who died of AIDS acquired *in utero*, and the final 3 were men who had died without any history of risk factors for AIDS. The CD4 counts of the men with AIDS ranged from 10 to 60. Pooled normal lymphocytes infected with HIV-1-IIIB for 4 days and sham-infected cells, kindly provided by Dr. Roy Steigbigel, were used as controls.[8] Depending on their size, one to three 4-μ paraffin-embedded sections were placed in order on sequentially labeled slides to facilitate comparison of HIV-1 nucleic acids and phenotypic studies on serial sections.

Viral Detection

Detection of PCR-amplified HIV-1 DNA was done using our previously published protocol.[8,9,11] Briefly, after protease digestion (2 mg/ml pepsin at room temperature for 30 minutes), PCR was performed using a solution that contained PCR buffer (GeneAmp kit; Perkin-Elmer Corp., Norwalk, CT), 4.5 mmol/L $MgCl_2$, 200 μmol/L dNTPs, 1.0 μmol/L of the primers (SK38/SK39 or SK145/SK431), 1 mg/ml of bovine serum albumin, and 2.5 U/25 μl of Taq DNA polymerase. The hot start modification increases the sensitivity of the assay to one copy with a single primer pair.[12] After an initial denaturing step of 94 C for 3 minutes, 30 cycles were completed using the following protocol: annealing/extension at 55 C for 2 minutes and denaturing at 94 C for 1 minute. After PCR, the probe (SK19 for SK38/39 and SK102 for SK145/431) at 50 ng/ml in a solution of 10% formamide, 5% dextran sulfate, and 300 mmol/L NaCl and amplified DNA were simultaneously denatured by heating to 100 C for 5 minutes. After the hybridization and wash steps, the slides are incubated in the anti-digoxigenin-alkaline phosphatase conjugate (1:50 dilution) at 37 C for 30 minutes and the probe/target complex detected by incubation in the chromagen NBT/BCIP nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) (ONCOR Corp., Gaithersburg, MD). A hybridization signal is evident as a dark blue precipitate, whereas the counterstain nuclear fast red stains nuclei and cytoplasm pale pink.

The procedure for reverse transcription *in situ* PCR has three modifications: 1) after protease digestion the tissue is treated overnight in a RNAse-free DNAse solution (Boehringer Mannheim, Indianapolis, IN; 10 U/section at 37 C) that eliminates nonspecific mispriming and DNA repair; 2) reverse transcription using the downstream primer (SK 39 or SK 431) is done at 42 C for 30 minutes according to the manufacturer's recommendations (Perkin-Elmer); and 3) direct incorporation of digoxigenin dUTP into the PCR product is accomplished by using 10 μ of the reporter molecule in the amplifying solution. Additional controls for the specificity of the signal with reverse transcription *in situ* PCR included the use of nonsense (HPV specific) primers, the omission of the reverse transcription step, and a pretreatment in an RNase solution.

Immunohistochemistry

To determine the phenotype of the cells, serial sections were incubated overnight at 4 C with either a mouse CD4 (1:10), leukocyte common antigen (1:40) (LCA, lymphocytes, and macrophages), or Mac 387 (1:500) (macrophages) monoclonal antibody (DAKO, Carpinteria, CA), as previously described (dilution in parentheses).[7,8,11]

Results

Forty-six tissues from the 18 males were analyzed for sequences homologous to HIV-1. The distribution of these 46 tissues were: testes 18, prostate 14, epididymus 8, seminal vesicles 3, and penis 3. Of these 46 tissues, 40 were from HIV-1-infected patients with the other 6 from the non-HIV-1-infected negative controls. Five of the 11 testicular tissues from men with AIDS showed retarded maturation of spermatogenesis where spermatid production was still evident. In the other six cases, relatively few spermatocytes and very rare to no spermatids were seen. In addition to the retarded spermatogenesis, these tissues showed varying degrees of fibrosis and atrophy that occurs in over 90% of men with advanced stage AIDS.[4,13] The testes from the other people were histologically normal.

The results of the analysis for HIV-1 DNA is provided in Table 1. Note that the provirus was detected by standard *in situ* hybridization in only 3/12 (25%) of the testes from the HIV-1-infected men and that the rate increased to 11/12 (92%) if the *in situ* hybridization was preceded by PCR. The one negative tissue showed Sertoli cells only. The distribution of the PCR-amplified viral DNA was equivalent when serial sections were compared using the SK 38 and 39 primers with the SK 19 probe and the SK 145 and SK 431 primers and the SK 102 probe, each corresponding to the gag region of the viral genome. Also note that each of the three testes from the non-HIV-1-infected people and the three children with AIDS were negative for PCR-amplified viral DNA. In the testes from men with HIV-1 infection, the virus localized to the spermatogonia, spermatocytes, and spermatids; 5 to 20% of the spermatogonia and spermatocytes were infected with approximately 1% of the spermatids HIV-1 positive (Figure 1). The virus was not detected in the Sertoli cells, Leydig cells, or endothelial cells which thus served as internal negative controls. The number of viral positive cells in the testes was inversely related to the degree of spermatogenesis maturation arrest. In the testes where spermatid production was still evident, hundreds of viral positive cells in a 1.5-cm tissue section were noted but only with PCR *in situ* hybridization; serial sections from such areas were either negative or showed a few positive cells when standard *in situ* hybridization was done (Figure 2). Conversely, in the testes in which Sertoli cells and rare spermatogonia were evident in the tubules, only rare viral positive cells were seen (Figure 1). PCR-amplified viral DNA was noted in rare stromal cells that cytologically appeared to be macrophages; analysis of serial sections showed that these cells did label with LCA and Mac 387. Scattered macrophages and lymphocytes in the testes were CD4 positive; no spermatogonia, spermatocytes, or spermatids contained detectable epitopes against the CD4 antibody.

Table 1. *Detection of HIV-1 in the Testes and Prostate*

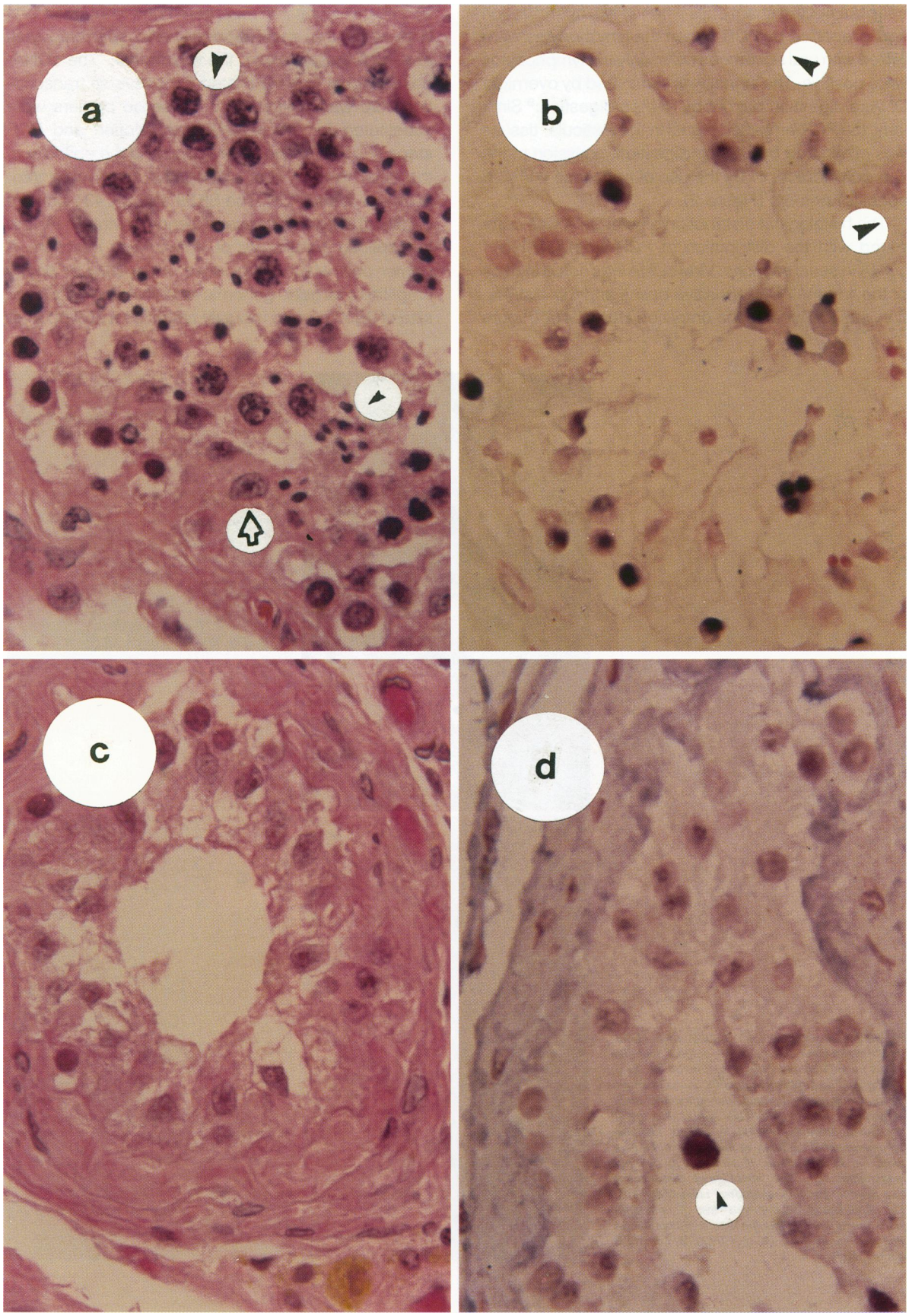
	HIV-1 DNA (ISH)	HIV-1 DNA (PCR ISH)	HIV-1 RNA Reverse Transcription *In Situ* PCR
HIV-1-infected men*			
Testes	3/12	11/12	11/12
Prostate	1/11	3/11†	2/11
HIV-1-infected children			
Testes	0/3	0/3	0/3
No HIV-1 infection: men			
Testes	0/3	0/3	0/3
Prostate	0/3	0/3	0/3

* 11/12 men had AIDS.
† Rare HIV-1-infected macrophages and lymphocytes were seen. PCR-amplified viral nucleic acids were not detected in the epithelia from the prostate, seminal vesicles, epididymus, or penis.

PCR-amplified viral DNA was detected in 3 of the 11 prostatic tissues (Table 1), in 1 of the 8 epididymal tissues, and in none of the tissues from the seminal vesicles or the penis from the HIV-1-infected men. The three positive prostatic tissues showed moderate to severe inflammation as evidenced by many macrophages and lymphocytes on histological examination and substantiated by analysis with LCA and Mac 387. Most of the viral-infected cells were in the lumen and were macrophages as determined by their cytological appearance and labeling with Mac 387. It should be stressed that only 1 to 5% of the macrophages and lymphocytes in these tissues were HIV-1 positive. Furthermore, the epithelium of the prostate and epididymus were invariably negative.

The specificity of the *in situ* DNA product was evaluated in the lymphocytes infected *in vitro* with HIV-1. A hybridization signal was not evident in either the HIV-1-infected or sham-infected cells using standard *in situ* analysis for HIV-1 DNA. A signal was evident, however, in over 95% of the HIV-1-infected

Figure 1. *Molecular analyses of testicular tissues from men who died of AIDS. This testes from a 37-year-old man with AIDS showed spermatid production (small arrowhead, spermatid; large arrowhead, spermatogonia and spermatocytes; open arrow, Sertoli cells)* (**a** *H&E); HIV-1 DNA was detected by PCR* in situ *hybridization and localized to many spermatogonia, spermatocytes, and to occasional spermatids (arrow-negative Sertoli cells)* (**b**). *The other testes is from a 44-year-old man in which only rare spermatogonia are seen among the Sertoli cells* (**c** *H&E). The rare PCR-amplified HIV-1 DNA containing spermatogonia in the seminiferous tubules reflects the marked arrest of spermatogenesis* (**d**). *Positive nuclei in* **b** *and* **d** *are dark due to the action of the alkaline phosphatase-anti-digoxigenin antibody conjugate on the chromagen NBT/BCIP; negative nuclei are pink due to the counterstain nuclear fast red (each ×400).*


a
b
c
d

cells but not the sham cells when *in situ* hybridization was preceded by PCR. When the lymphocytes were mixed with oral squamous cells, only the lymphocytes were positive. The signal was eliminated by overnight DNAse digestion but not by RNAse digestion.[8] Similarly, the PCR-amplified signal in the testicular tissues was eliminated by DNAse digestion, omission of the Taq polymerase, or by the use of nonsense (HPV 18 specific) primers (Figure 2).

The histological distribution of viral DNA was compared with the distribution of PCR-amplified HIV-1 cDNA in serial sections. Viral cDNA was noted in each of the 11 HIV-1 DNA-positive cases and the distribution was identical to the amplified HIV-1 DNA (Figure 2). The cell types that most commonly contained viral RNA were the spermatogonia and spermatocytes and, to a lesser extent, the spermatids. The cDNA-based signal was eliminated if the reverse transcription step was omitted or if nonsense primers were substituted for the reverse transcription and PCR steps (Figure 2). The other testes and the epithelia of the other tissues were all negative by reverse transcription *in situ* PCR. The SK39 and SK 431 primers cannot distinguish between genomic viral RNA and the spliced transcripts of active viral infection. A primer pair, which either does not amplify or poorly amplifies a target in genomic viral RNA due to its large size of 3500 bp and robustly amplifies a 250 bp after

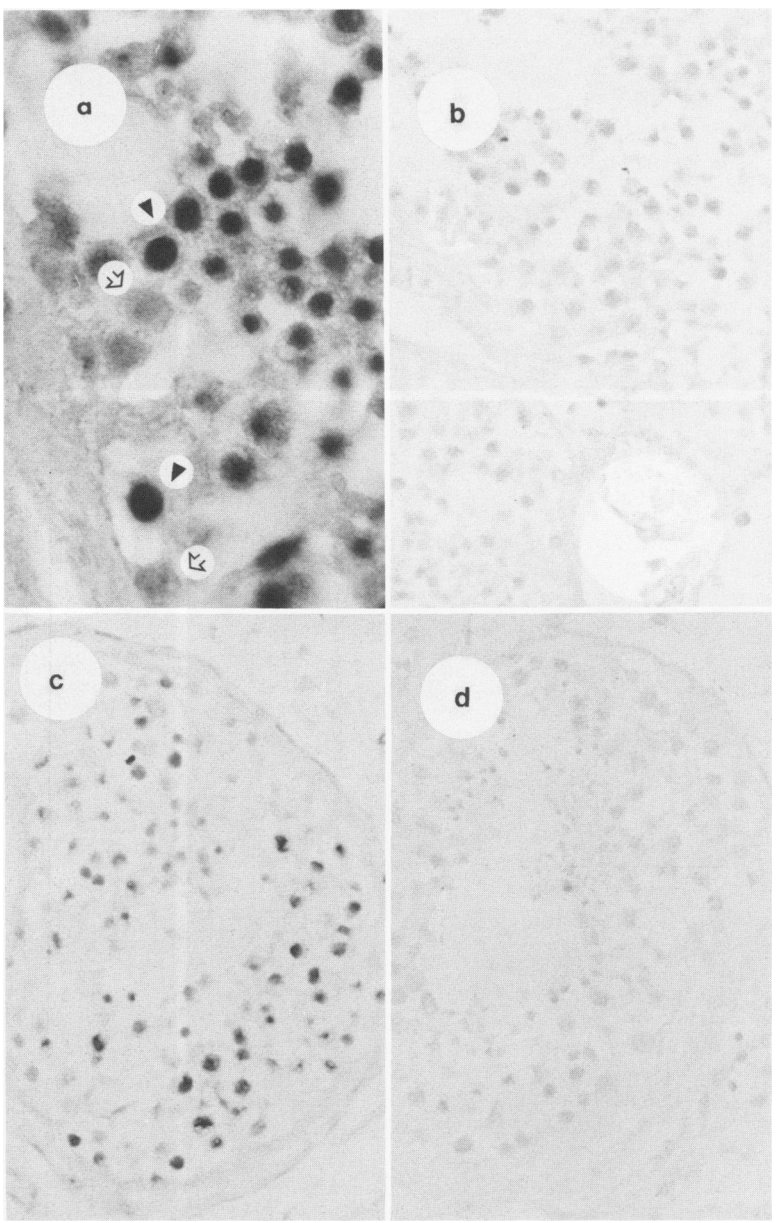


Figure 2. *Detection of HIV-1 DNA and cDNA in the testes with controls. HIV-1 DNA was detected by PCR* in situ *hybridization in the spermatogonia and their progeny* (**a**, *closed arrowhead, spermatogonia and spermatocytes; open arrowhead, Sertoli cells*). *The signal was lost if the tissues were pretreated in DNase, the Taq polymerase was omitted, nonsense primers were used, or, in this tissue, if standard* in situ *hybridization* (**b**). *The histological distribution was similar for PCR-amplified viral cDNA* (**c**). *The RNA-based signal was lost if nonsense primers were used or if the reverse transcription step was omitted* (**d**) (**a** *is* ×*1000;* **b**, **c**, *and* **d** *each* ×*400*).

splicing in the rev and tat exons (kindly provided by Dr. Roger Pomerantz), produced an intense signal after reverse transcription *in situ* PCR in most of the cells that showed a signal using the other primer pairs.

Discussion

This study analyzed the histological distribution of PCR-amplified HIV-1 DNA and RNA in the male genital tract. It was demonstrated that HIV-1 DNA was detected in the testes of over 90% of men with the viral infection. However, in most cases the virus was only detectable if the *in situ* hybridization analysis was preceded by PCR amplification. This is consistent with other studies that examined the cellular distribution of the HIV-1 provirus in a variety of sites including the uterine cervix, lymph nodes, peripheral blood leukocytes, and the central nervous system.[5–11,14] There was a striking localization of the PCR-amplified viral DNA to the spermatogonia and their progeny. The infection was marked by the production of genomic and multiply spliced viral transcripts as determined by reverse transcription *in situ* PCR, which is equivalent to the productive infection in infected cell lines such as CR10 and H9.[7,8]

The findings in this study may explain several observations about HIV-1 disease of the male genital tract. The marked atrophy, fibrosis, and arrest of spermatogenesis typical in the testes of men with AIDS may reflect direct HIV-1 infection of the spermatogonia and their progeny with their ultimate death.[3,4,13] Further study to address this issue should include *in vitro* infection of spermatogonia-type cell lines. The observation that the spermatogonia and their progeny are actively infected by HIV-1 also may explain the mechanism whereby the virus is transmitted by sexual relations. Sexual transmission of HIV-1 is the primary mode of spread of the virus on a world-wide basis. Active infection of spermatogonia with production of infectious virions would explain that up to 57% of semen samples from men with AIDS contains infectious viral particles, as demonstrated by co-culturing with peripheral blood lymphocytes.[13] The infectious particles could be released from intact or degenerated spermatogonia and spermatocytes. Interestingly, a much lower percentage of spermatids in the testes were HIV-1 positive compared with the spermatogonia and primary spermatocytes, suggesting that cellular degeneration was associated with the terminal meiotic event. Pomerantz and Bagasra has shown using *in situ* PCR that HIV-1 is present in on average 1:500 spermatids in semen, which is similar, although somewhat lower, to the rate noted in spermatids in the testes in this study (R. Pomerantz and O. Bagasra, personal communication). Shofield et al[15] noted that HIV-1 could be detected in the spermatid fraction of semen by solution phase PCR in contrast to other studies[2] and suggested that semen contains factors that relate to lipoperoxidation, that inhibits PCR amplification which, when inactivated, allow for detection of the virus in the spermatid fraction by solution phase PCR. We speculate from the findings in this study that venereal transmission of HIV-1 from the male relates primarily to infectious particles released from infected and degenerating spermatogonia and spermatocytes. Direct infection of spermatids may be a contributing factor but probably much less important, as suggested by the low rate of infectious viral particles in the spermatids in semen as determined by co-culturing experiments.[13] Further study that should include correlation of viral infection in the semen and testes is needed to address this issue.

It is well documented that women are at a 10 times greater risk then men to acquire HIV-1 infection via heterosexual relations.[1,8] It has been shown that the target cell in the female genital tract is the macrophage that localizes to the 1-cm transformation zone where the squamous and columnar epithelium merge.[8] With heterosexual sex these cells and their mucosal counterpart, the Langerhans cell, would be directly exposed to HIV-1 virions. Alternatively, the equivalent zone in the male genital tract, the penile urethra, is very small and much less active, containing far fewer macrophage-type cells (G.J. Nuovo, unpublished observations). The male counterpart of the transformation zone in women is the anorectal junction, which could explain the higher incidence of HIV-1 transmission with male to male anal intercourse. The target cell in the male genital tract, the spermatogonia, would not be directly exposed to the virus via sexual transmission, which may explain the more smaller relative risk to men of acquiring AIDS via heterosexual relations.

Spermatogonia infection probably occurs secondary to hematogenous spread of the virus and may have its histological correlate in the viral-infected macrophages that were detected in the interstitium of the testes in this study. However, direct infection from infectious virions in the peripheral blood is a distinct possibility given the highly vascular nature of the testes. HIV-1 may bind to and internalize into spermatogonia via the CD4 antigen that has been reported on these cells, although alternative receptors, such as galactosyl ceramide, may also be important;[16,17] we did not detect the CD4 receptor on

the spermatogonia in this study. Further study is needed to determine whether the uniform absence of PCR-amplified HIV-1 nucleic acids in the testes of prepubertal boys is due to the lack of a viral receptor or some other mechanism.

Spermatogonia in adults need to be included in the list of cells with the T helper cell and the macrophage that is a primary target of the viral infection. Spermatogonia infection clearly offers the teleological advantage for viral venereal spread, especially in early stage disease when spermatogenesis is robust and many spermatogonia and their progeny are present. Of interest in this regard is the one case of an asymptomatic HIV-1-infected man who had many HIV-1-positive spermatogonia and spermatocytes and could thus be transmitting the disease before any clinical evidence of AIDS. Further study of the mechanisms by which HIV-1 infects the spermatogonia, the consequences to these cells of this infection, and the mechanisms of how the virus is transmitted to the cervical macrophages will be critical toward control of the venereal spread of HIV-1 infection.

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