HIV-Particles in Spermatozoa of Patients with AIDS and Their Transfer into the Oocyte

Baccio Baccetti,* Arrigo Benedetto,‡ Anna G. Burrini,* Giulia Collodel,* Elvira Costantino Ceccarini,* Nicla Crisà,§ Antonino Di Caro,* Mariela Estenoz,* Anna R. Garbuglia,‡ Adina Massacesi,§ Paola Piomboni,* Tommaso Renieri,* and Delia Solazzo*

*Institute of General Biology and Center for the study of Germinal Cells, Consiglio Nazionale delle Ricerche, 53100 Siena, Italy; ‡Center of Virology, S. Camillo Hospital, 00152 Roma, Italy; and §"Cristo Re Hospital," 00152 Roma, Italy

Abstract. By immunocytochemistry and in situ hybridization at the electron microscopy level, and by the PCR technique, we have shown that HIV-1 binds and enters normal sperm; that viral particles, their antigens, and nucleic acid are present in sperm from HIV-1 infected men; and that such sperm can transfer HIV-1 like particles to normal human oocytes. We also present evidence that a galactosylceramide-like compound is present on the sperm membrane and could function as an alternative receptor for HIV.

THE possibility that HIV is carried by sperm has been controversial. Using electron microscopy, some investigations have detected HIV-like particles in the sperm of infected individuals and in virus-incubated normal sperm (Bagasra et al., 1988, 1990; Bagasra and Freund, 1990; Baccetti et al., 1990, 1991, 1992; Scofield, 1992). Others (Pudney, 1990; Anderson et al., 1990; Anderson, 1992) have failed to confirm these findings. This controversy also involved the detection of the CD4 receptor on the surface of human spermatozoa. Some investigators were able to demonstrate the presence of such a receptor (Bagasra et al., 1988, 1990; Bagasra and Freund, 1990; Gobert et al., 1990), and the same conclusion was indirectly reached by Ashida and Scofield (1987), Miller and Scofield (1990), Scofield (1992), and Scofield et al. (1992*a*), while others failed to identify CD4 (Wolff et al., 1988; Anderson et al., 1990; Anderson, 1992). Similarly, although sperm were found in one study to contain HIV-1 DNA (Scofield et al., 1992*b*) others report that sperm are negative for viral nucleic acid (Mermin et al., 1991; Van Voorhis et al., 1991). Using immunoelectron microscopy, in situ hybridization, and PCR analysis, we show here that HIV-1 binds and enters sperm, both in vitro and in vivo, and that virus particles transferred by such sperm to eggs are present both in the zygote and in the blastomeres of early embryos.

Materials and Methods

Case Histories

The 30 seropositive semen donors for this study were from a group of HIV-1-infected men who were intravenous drug users. At the time of semen donation, all were viremic (as assessed by the presence of p24 antigen in the blood) and had T4 lymphocyte counts of <400/mm^3 (averaging 280 ± 60 SEM for the group). Each had generalized lymphoadenopathy with fever and/or weight loss. These symptoms and clinical findings classify the disease stage of the donors as CDC (Center for Disease Control, Atlanta, GA) class 4th A. Although half of the donors were azoospermic, semen from the remaining 15 contained motile spermatozoa. HIV-1 particles were found in the spermatozoa (selected after swim up and Ficoll) of eight of these patients (see below).

Address all correspondence to Prof. Baccio Baccetti, Institute of General Biology and Center for the Study of Germinal Cells, Consiglio Nazionale delle Ricerche, Via T. Pendola, 62, Siena, Italy.

Production and Titration of the Viral Stocks

The supernatant from H9/HTLV IIIB cells, kindly provided by Prof. G. B. Rossi (Istituto Superiore di Sanità, Rome, Italy), was used as a source of infecting virus (Benedetto et al., 1993).

The H9/HTLV IIIB cell line was maintained in RPMI 1640 with 10% fetal calf serum (RPMI) at 37°C in a CO_2 atmosphere and was passaged every 5 d at 1:5 split ratio. Medium from exponentially growing cells was collected, filtered through 0.45-μm filters (Millipore Corp., Bedford, MA), and used as virus stock (HIV-1). The titration of HIV/H9 stocks was performed in 96-well flat-bottomed plates (Falcon Labware, Cockeysville, MD). A sample (0.1 ml) of 10-fold dilutions of virus stocks (10 wells for each dilution) was added to wells containing 5×10^4 C8166 T cells in 0.1 ml. The cultures were incubated and monitored daily for ≤120 h. The wells were scored as positive when at least one syncytium could be observed. Virus titers, expressed as tissue culture infective dose 50% ($TCID_{50}$), were calculated according to the formula of Reed and Muench (1938).

Infection In Vitro of Spermatozoa from Healthy Donors

2 ml of HIV-1 stock ($10^{4.7}$ $TCID_{50}$) were added to 2 ml of sperm from healthy donors and incubated at 37°C in 5% CO_2. The experiment was carried out on 15 donors. After 5 h, the sperm cells were collected and processed for biochemical, immunocytochemical, and electron microscopical studies, as described in the corresponding sections. Uninfected and HIV-1 infected (72 h after infection) C8166 T cells were used as negative and positive controls. In all the experiments, spermatozoa containing virions were seen. Two additional experiments were carried out by cocultivating the spermatozoa of two healthy donors for 5–12 h with 72-h infected C8166 T cells. The mixed cultures were fixed and embedded for electron microscopy as described in the subsequent section.

Electron Microscopy

Ultrastructure. Sperm from seronegative donors that were incubated in vitro with HIV-1 or with infected C8166 T cells were washed, collected by centrifugation, and the pellet was fixed for 2 h at 4°C in Karnovsky fixative, rinsed overnight in cacodylate buffer, postfixed in 1% buffered OsO_4, dehydrated, and embedded in Epon Araldite. Motile spermatozoa obtained from HIV-1–infected patients and selected after swim up and Ficoll were fixed and embedded as described above. As negative controls, sperm obtained from seronegative donors were treated using the same procedure. Ultrathin sections were cut using a Supernova Reichert-Jung (Wien, Austria) ultramicrotome, and were then stained with uranyl acetate and lead citrate. Observation and photography were performed using transmission electron microscopes (EM 301 and CM10; Phillips Electronic Instruments Co., Mahwah, NJ). Magnification was calibrated in each section on the diameter of microtubules, and by using Balzers (Fürstentum, Lichtenstein) Cross Gratings of 54,000 lines/inch (d = 0.463 μm). Healthy or more or less faulty spermatozoa of several hundred seronegative men examined in this laboratory during the last 10 years have been used as controls.

The time course of the viral binding was detected as it follows. Spermatozoa from two seronegative donors were incubated with HIV-1 for 2 h at 4°C, and were then transferred for an additional 4 h at 37°C; an aliquot of each sperm sample was drawn after 2 h, and after 6 h of incubation, washed twice, centrifuged, and the pellet was processed for electron microscopy as described above.

Immunocytochemistry. Aliquots of sperm from the 15 HIV-infected patients, from the 15 seronegative donors incubated with HIV-1, or infected C8166 T cells were fixed for 1 h at 4°C in 1% glutaraldehyde or 3% *p*-formaldehyde/0.1% glutaraldehyde in PBS, rinsed overnight in PBS/5% sucrose, and incubated for 30 min in PBS containing 50 mM NH_4Cl. After centrifugation, the pellets were embedded in 3% agar/PBS, dehydrated, and embedded in Lowicryl K4M (Balzers) at −35°C according to Carlemalm et al. (1985). Sections cut from the embedded blocks were mounted on formvar-coated nickel grids, incubated for 2 h at room temperature or overnight at 4°C with antibodies (anti-p24 monoclonal antibody, Technogenetics, SeraLab, or DuPont deNemours diluted 1:40 in PBS containing 0.1% BSA and 1% normal goat serum, or pooled sera from AIDS patients diluted 1:100 in PBS containing 0.1% BSA), washed three times in PBS 0.1% BSA, and incubated for 1 h at room temperature either with anti-mouse IgG conjugated with 10-nm colloidal gold particles (Amersham International, Amersham, U.K.) (anti-p24 incubated sections) diluted 1:50 or protein A conjugated with 10-nm colloidal gold particles (Amersham International) (sections incubated with human sera) diluted 1:50. After incubation, the sections were rinsed, stained, and observed as described above. To control for nonspecific binding by the anti-mouse IgG and protein A secondary reagents, some sections were carried through a staining procedure that was identical in every respect, except that the primary antibodies were omitted. Untreated sperm from 10 healthy donors and uninfected C8166 T cells served as controls for nonspecific binding by monoclonal anti-p24 and immunoglobulins from the pooled human sera.

In Situ Hybridization

Biotin-labeled DNA Probes. A 9.0-kb HIV-1 DNA probe (Oncor, Gaithersburg, MD) and a mixture of SK31, SK19, and SK70 oligonucleotides (representing different internal regions of the HIV-1 *LTR*, *gag*, and *env* regions, Ou et al., 1988), were end-labeled with Biotin-dUTP and terminal deoxynucleotidyl transferase (Boehringer Mannheim GmbH, Mannheim, Germany). The labeled oligonucleotides were purified by gel filtration on Sephadex G-25 DNA grade (Pharmacia Fine Chemicals, Uppsala, Sweden), concentrated by evaporation, and then suspended in hybridization buffer containing 4× SSC (0.6 M NaCl and 0.06 M sodium citrate), 50% freshly deionized formamide, 10% dextran sulfate, and 1 mg/ml salmon sperm DNA. The labeled probes were stored at −20°C.

Hybridization. Ultrathin sections of the Lowicryl-embedded sperm from the 15 HIV-infected patients, from the 15 in vitro–infected healthy donors, and in vitro–infected or uninfected C8166 T cells were mounted on nickel grids. Oligonucleotide probes were denatured for 3 min in boiling water, immediately cooled at 0°C, and added to the hybridization solution (at 10–20 μg/ml). After treatment of the grids with proteinase K (10 μg/ml), the grids were incubated in hybridization solution. Incubation was performed according to Fournier et al. (1991), by floating the grids on 3–4-μl drops of hybridization solution for 16 h at 37°C in a moisture chamber. After incubation, the grids were washed sequentially in 50% formamide/4× SSC (2 × 5 min at 37°C), 4× SSC (2× 5 min at 37°C), 0.2× SSC (2 × 5 min at room temperature), distilled water (2× 2 min at room temperature). Control sections from uninfected material were treated in the same way, while control sections of infected material were treated identically, but with the DNA probes omitted.

Visualization of the Hybridized Probe. After hybridization washings, sections were sequentially incubated for 15 min in PBS/1% BSA and for 1 h in 1% rabbit IgG antibiotin (Enzo Biochem Inc., Inc. New York), then rinsed twice for 10 min in PBS/0.1% BSA/0.05% Tween 20, and kept in contact for 1 h on a drop of anti-rabbit IgG conjugated with 10-nm colloidal gold particles (Amersham International) diluted 1:50. Subsequently, the grids were rinsed twice in PBS/BSA/Tween 20 and distilled water, and were then stained with uranyl acetate.

Detection of HIV-1 RNA and HIV-1 Proviral DNA in Spermatozoa by Polymerase Chain Reaction

Spermatozoa Purification. Semen from two infected donors and one healthy man was centrifuged, and 1 ml of RPMI was layered onto the pellet. Samples were then incubated at 37°C in CO_2 incubator for 30 min, at which time, motile spermatozoa that had swum from the pellet into the overlying medium were collected, washed in PBS, layered over a Ficoll cushion (Lymphoprep; Nycomed Pharma, Oslo, Norway), and centrifuged at 700 *g* for 20 min at room temperature. The pelleted spermatozoa were then suspended in RPMI containing 5% FCS and subjected to two more Ficoll separations as described above.

The last pellet, which consisted of 100% motile spermatozoa, was then washed three times in PBS (purified spermatozoa fraction). An aliquot of this fraction was smeared onto a slide, and another was processed as a pellet for electron microscopy to confirm the absence of cell types other than spermatozoa, and to evaluate the ultrastructural characters that influence fertility power, according to the formulas of Baccetti and Mirolli (1994) and Baccetti et al. (1994*a* and 1994*b*).

After each pelleting of the sperm through Ficoll, the entire layer of cell-free medium overlying the Ficoll cushion was collected (Ficoll supernatant fraction), as were any cells sedimenting at the top of the Ficoll cushion (Ficoll fraction). After the final Ficoll separation, the three supernatant fractions were pooled and centrifuged. The three interface layers were also pooled, diluted 1:5 in RPMI/5% FCS, and centrifuged. Visual inspection disclosed that this cell suspension contained small numbers of spermatozoa, immature germinal cells, mononuclear leukocytes, and cell debris.

Detection of HIV-1 RNA by RT-PCR. RNA was extracted from the three fractions by the guanidine thiocyanate method (Chomczynski and Sacchi, 1987). After treatment with DNAse I, the RNA samples were dissolved in 30 μl of diethylpyrocarbonate-treated H_2O containing 20 U of RNAsin (Diethylpyrocarbonate was from Sigma Immunochemicals, St. Louis, MO). 5 μl of each RNA solution, heated at 70°C to denaturate RNA molecules, were reverse transcripted in 20 μl of a mixture containing 50 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD), 0.2 μM SK39 primer, and 0.2 mM each of the four deoxyribonucleoside triphosphates (the four dNTPs were suspended in 50 mM Tris-HCl, pH 8.3, 75 mM KCl, and 3 mM $MgCl_2$). The reaction was allowed to proceed for 1 h at 37°C.

Amplification of reverse-transcripted RNA was performed in a DNA thermocycler (Perkin-Elmer Corp., Norwalk, CT), using oligonucleotide primers corresponding to a region within the HIV *gag* gene (SK38 and SK 39, Ou et al., 1988). 100 μl of reaction mixture (containing 10 μl of reverse transcription product, 50 mM KCl, 10 mM Tris, pH 8.3, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 200 μg/ml of gelatin, 2 U of Amplitaq DNA polymerase [Perkin Elmer Corp.], and 0.5 μM of the SK38/39 primers) were subjected to 30 cycles of amplification with the following program: 2 min at 94°C, 90 s at 55°C, and 2 min at 72°C. 5 μl of the amplified mixture were subjected to a second round of PCR as described above.

Aliquots of each amplified sample were electrophoresed in a 3% NuSieve/1% Sea Kem GTG agarose (IMC Bioproducts, Rockland, ME) gel in TBE buffer (90 mM Tris, pH 8.0, 90 mM boric acid, and 2 mM EDTA) containing ethidium bromide. The resulting bands were transferred to a Hybond N^+ membrane filter (Amersham International, Amersham, U.K.) with 0.4 N NaOH under a slight vacuum. After prehybridization of the filter (4× SSC, 5× Denhardt's solution, 0.5% SDS, and 10 μg/ml of denatured, sonicated salmon sperm DNA for 2 h at 55°C), hybridization was carried out overnight under the same conditions, but in the presence of 10 pmol of ^{32}P end-labeled SK 19 probe (specific activity = 2–5 × 10^9 cpm/pmol). After washing for 15 min at room temperature in 2× SSC/0.1% SDS, the membranes were then autoradiographed at −70°C on Chronex-4 film with intensifying screen (Du Pont Co., Wilmington, DE).

RNA extracted from 1 ml of the culture medium of HIV-1–infected C8166 T cells ($10^{5.2}$ $TCID_{50}$) after treatment with DNAse I served as HIV RNA positive control.

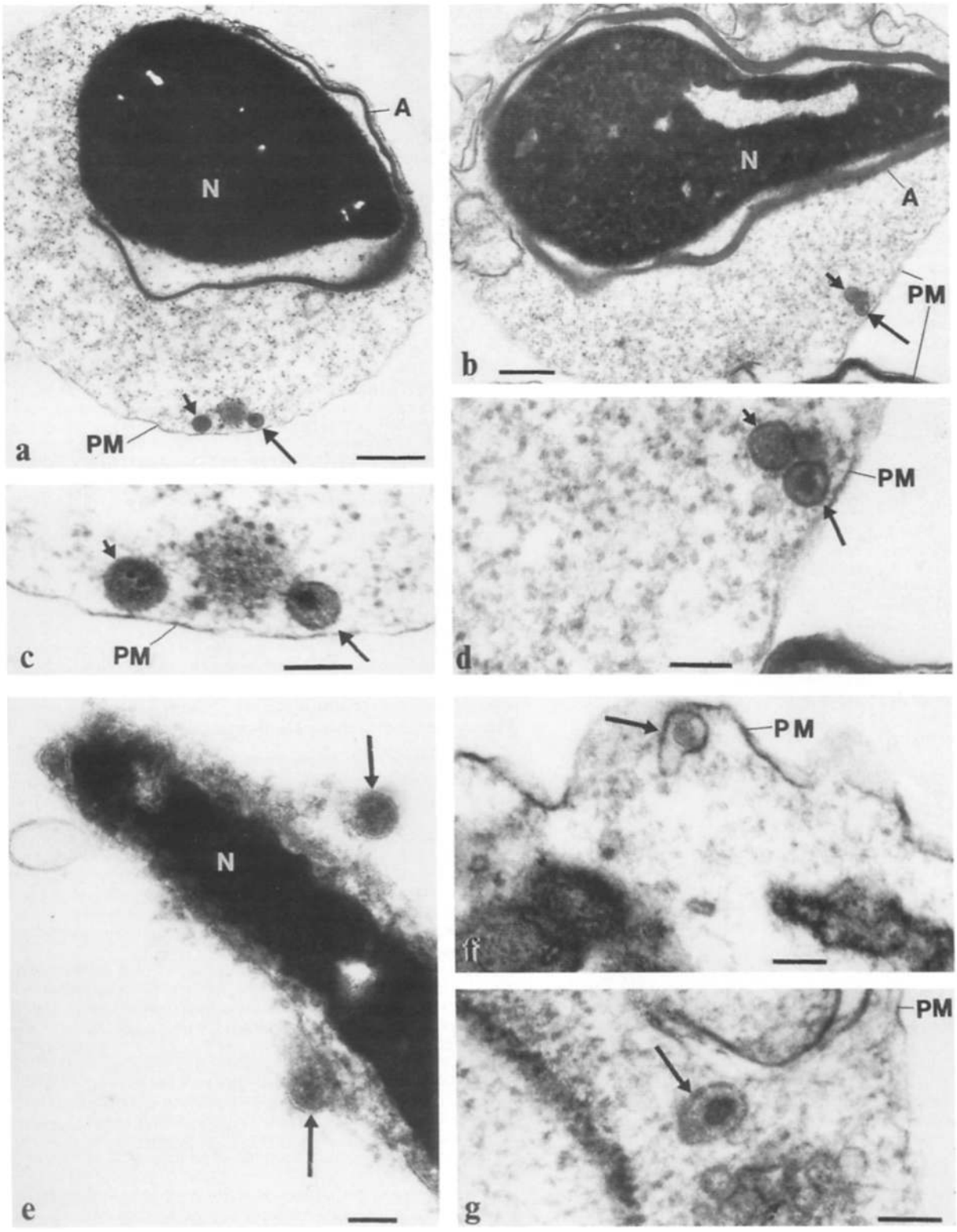
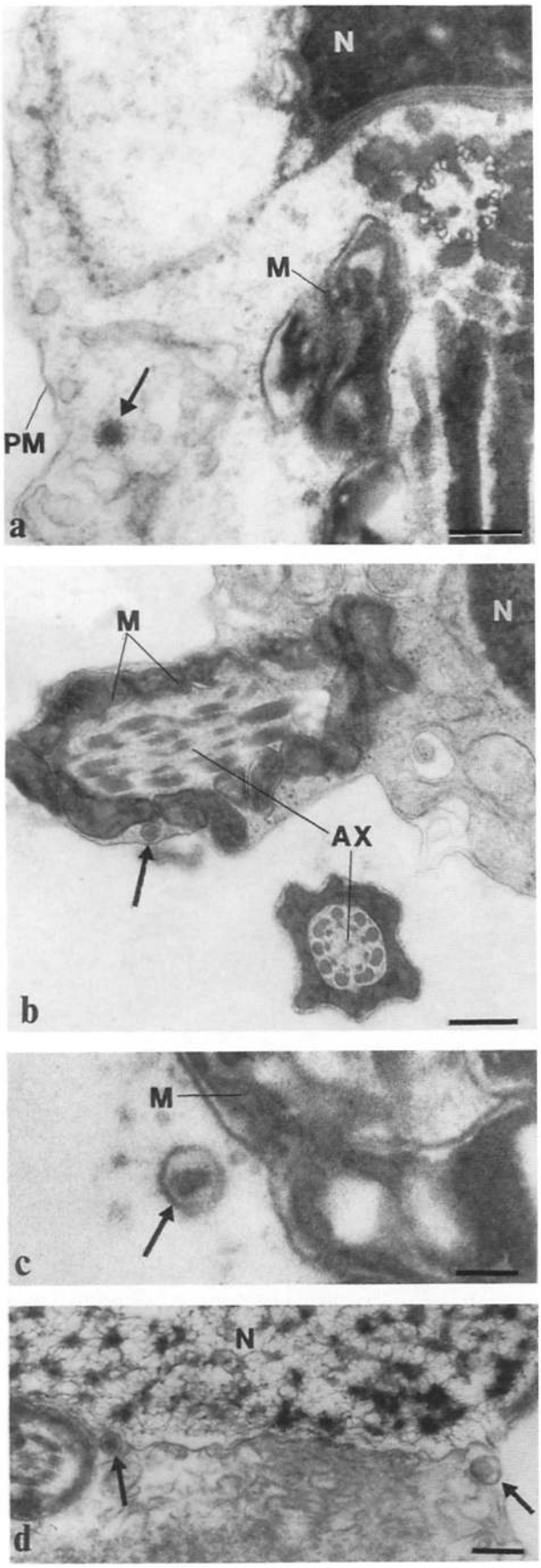


Figure 1. Transmission electron micrographs of HIV-like particles (*arrows*) in the cytoplasm of spermatozoa from HIV infected patients. (*a–e*) Cytoplasm surrounding the sperm nucleus. The particles pointed to by long arrows are surrounded by a membrane-like coat and show the typical electrondense nucleoid. The particles pointed to by short arrows are full of granular material and devoid of nucleoid. *c* and *d* are enlargements of *a* and *b*, respectively. In *e*, some virus particles devoid of nucleoids seem to bud out the perinuclear region (*arrows*). *f* and *g* Some viral particles with elliptic or pyriform coat with eccentric nucleoid (*arrows*) are present in the midpiece sperm region. *A*, acrosome; *N*, nucleus; PM, plasma membrane. Bars, 0.50 μm in *a*, 0.30 μm in *b*, 0.15 μm in *c*, and 0.10 μm in *d–g*.



Detection of HIV-1 Proviral DNA. A part of the purified spermatozoa fraction was lysed for 1 h at 60°C in 100 μl of lysis buffer containing 10 mM Tris, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.45% NP-40, 0.45% Tween-20, 60 μg/ml proteinase K, and 40 mM DTT. After inactivation of the proteinase K (by incubation at 95°C for 10 min), the mixture was centrifuged for 5 min at 10,500 *g*, and 10 μl of the supernatant was subjected to PCR for amplification of HIV-1 *gag* and *env* regions using SK38-39 and SK68-69 primers, respectively (Ou et al., 1988), as described in the reverse transcription PCR (RT-PCR)[1] paragraph. Lysates from uninfected and HIV-1-infected C8166 T cells were used as negative and positive controls, respectively. To control for the PCR procedure, aliquots of each cell lysate corresponding to 10^4 cells (spermatozoa or T cells) were also amplified using the PCO_3 and PCO_4 primers spanning a region within the beta-globin gene (Saiki et al., 1986). The sensitivity of our PCR protocol was determined by the amplification of 10-fold dilutions of an 8E5/LAV cell lysate. These cells contain a single integrated copy of HIV DNA (Folks et al., 1986). The minimal number of cells giving positive PCR signals ranged from 5 to 20. Similar values were obtained when 8E5/LAV cells were subjected to Ficoll gradient before the PCR assay.

In Vitro Fertilization

Mature oocytes were offered by HIV-1 seronegative women who had been superovulated in preparation for artificial insemination. Single oocytes were suspended in Menezo B2 culture medium (Biomerieux, Marcy l'Etoile, France) in organ culture dishes (no. 3037; Falcon Labware). Motile sperm from HIV-1-infected patients were purified by the procedure described above (see "Spermatozoa Purification"), an aliquot was fixed and tested by electron microscopy as described (see "Ultrastructure"), the remaining aliquot was added to the oocytes. After 18–20 h of incubation, fertilized oocytes were transferred into Menezo medium containing streptomycin and penicillin, and they were examined to confirm that two pronuclei were present. After removal the cells of the contracted corona, the zygotes were then incubated for 24 h. The resulting preembryos (segmented to contain eight blastomeres each) were then fixed, embedded in agar, and processed for electron microscopy as described above. Healthy oocytes and preembryos at the same stages were fixed and examined as control.

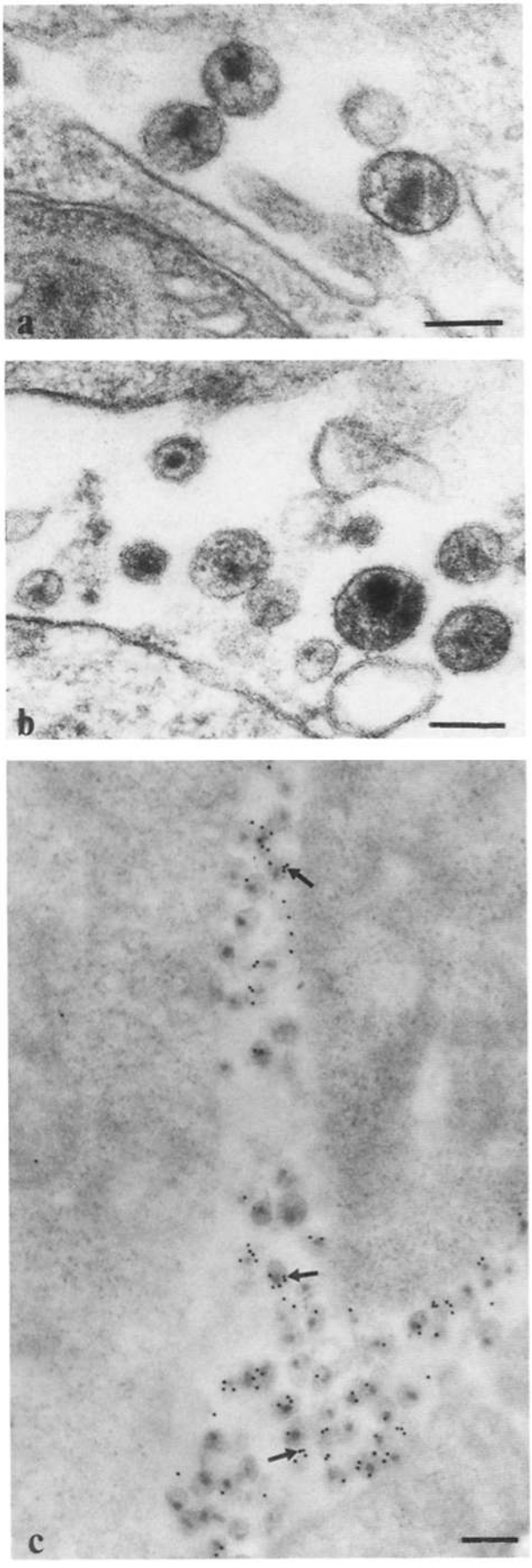
Fluorescence Immunocytochemistry

Aliquots of 1 ml of semen obtained from healthy individuals were overlaid with an equal volume of DME and incubated for 30 min at 37°C in a CO_2 incubator. The motile spermatozoa were collected from the overlaid medium, washed in PBS, and incubated for 20 min in PBS containing 3% normal serum from the species where the second conjugated antibodies were raised. The antigalactocerebroside, monoclonal 01 (IgM) (Sommer and Schachner, 1981), was provided by Dr. G. De Vries (Medical College of Virginia, Richmond, VA), and was prepared from the supernatant fractions of the hybridoma cells; the anti-IL-1 antibodies, used as negative control for the isotype (IgM), were a gift from Dr. Paolo Ghiara (Biocine, Siena, Italy).

Aliquots of 1×10^6 spermatozoa were incubated in 100 μl of mAb 01 diluted in 1:50 or in 100 μl of anti-IL-1 antibodies (1:10) for 20 min on ice, as described by Bensal et al. (1989). After incubation, the samples were diluted to 1 ml with PBS and centrifuged, the washing was repeated two more times, and bound antibodies were revealed by incubation with a secondary antibody (1:64) FITC-IgM μ chain (Sigma Immunochemicals, St. Louis, MO). After washing, the spermatozoa were smeared on glass slides, postfixed in 4% *p*-formaldehyde in PBS, and mounted in glycerol containing 5% *n*-propylgallate. In some experiments, the spermatozoa were fixed before incubation with mAb 01. Schwann cell clonal cell line A6 isolated in our laboratory and Swiss 3T3 cells grown on coverslips were used as posi-

1. *Abbreviations used in this paper*: RT-PCR, reverse transcription PCR; $TCID_{50}$, tissue culture infective dose 50%.

Figure 2. HIV-like particles (*arrows*) in the cytoplasm surrounding the initial region of the midpiece (*a–c*) or juxtaposed on the plasma membrane (*d*) from seronegative donors spermatozoa (*a–c*) and spermatid (*d*) incubated with HIV-1. The particle in *a* is devoid of membrane-like coat, which, on the contrary, surrounds the electron-dense core (*arrows*) of the particles in *b–d*. *Ax*, axoneme; *M*, mitochondria; *N*, nucleus; *PM*, plasma membrane. Bars, 0.15 μm in *a*, 0.30 μm in *b*, 0.10 μm in *c*, and 0.20 μm in *d*.



tive and negative controls, respectively, for the mAb 01. Fixing and incubation conditions were as reported above. Controls for secondary antibodies were carried out by omitting the primary antibodies. The samples were photographed with a light microscope (Aristoplan; E. Leitz, Rockleigh, NJ) equipped with fluorescence optics.

Results

Electron Microscopic Identification of HIV-1 in Sperm

Transmission electron micrographs of sperm from infected patients (Fig. 1, *a–g*) and of seronegative donor sperm that had been incubated with HIV-1 (Fig. 2, *a–d*) or cocultured with infected C8166 T cells, show sperm-associated retrovirus-like particles similar to those visible among C8166 T cells after incubation with HIV-1 (Fig 3, *a–b*). Particles were present as full-size virions located inside the sperm cytoplasm (Figs. 1, *a–d* and *g*, and 2, *a–c*) or attached to the sperm surface (Figs. 1 *e* and 2 *d*).

In in vitro–infected spermatozoa, these particles are present in ~30% of the examined sperm sections, and usually no more than one in a section. In the sperm of the infected patients, the number of particles seems to be quite minor (<1/10) than in HIV-1–incubated healthy spermatozoa. These numbers are limited to the particles sectioned through their maximal circumference and showing a recognizable nucleoid. For this evaluation, we have examined several hundreds of grids in three years. Obviously, the real number of particles is higher.

These particles belong to three different models. The more common and recognizable category is that of an outer dense coat having the aspect of a membrane vesicle, surrounding a central electron-dense nucleoid (Figs. 1, *a–d, f,* and *g*, and 2, *b–d*). The outer coat is a spheroid or pyriform wall, usually of 100–150 nm in the maximal diameter, made up of a 7.5-nm thick membrane (Figs. 1, *a–d, f,* and *g*, and 2, *b–d*). The osmiophilic dense nucleoid is located in more or less central position. It is shaped as a truncated cone or a cylinder having a basal diameter of 50 nm (Figs. 1, *c, d, f,* and *g*, and 2, *b–d*) and a height of 80–90 nm (Fig. 2 *c*). In the space between the nucleoid and the membrane, clumps of a granular material are contained, mainly close to the inner surface of the membrane (Fig. 1, *c, d,* and *g*). The second kind of particle is represented by a spheroidal wall, 100–120 nm in diameter, similar to that of the previous category, but devoid of nucleoid and full of homogeneous granular material (Fig. 1, *c* and *d*). These particles appear sometimes as budding from the nuclear region (Fig. 1 *e*). The third category of particles, very common in the sperm of infected patients or in seronegative donor sperm incubated with HIV-1, is that of dense cylindrical or truncated cone-shaped dense particles having a basal diameter of 50 nm and a height of 80 nm. These particles suggest the aspect of uncoated viral nucleoids inside the sperm cytoplasm (Fig. 2 *a*). We observe, in

Figure 3. Sections of HIV-1–infected C8166 T cells. (*a* and *b*) Several HIV-1 particles, with different diameters according to the plane of section and with the typical dense core, are visible among the cells. After immunogold labeling with anti-p24 monoclonal antibody (*c*), gold particles (*arrows*) are specifically localized on the extracellular virions. Bars, 0.10 μm in *a* and *b*, and 0.20 μm in *c*.

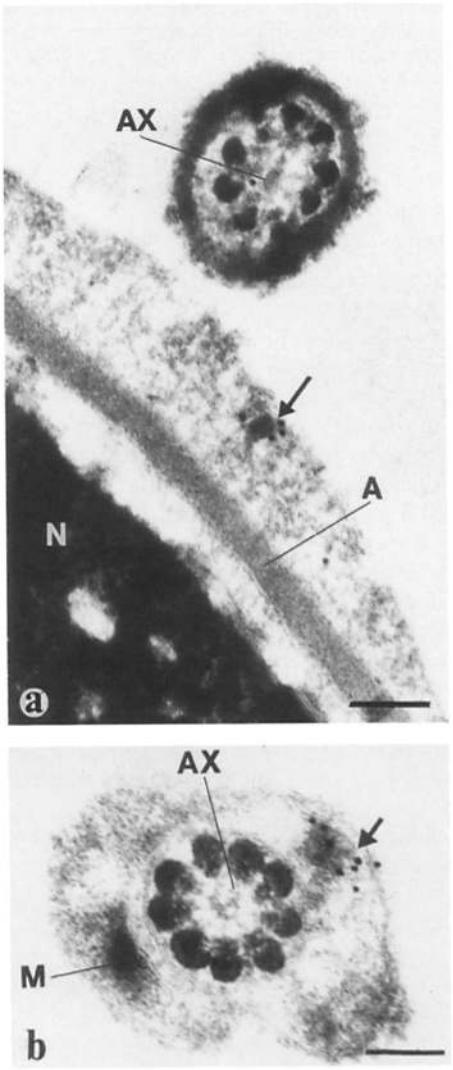


Figure 4. Submicroscopic immunogold detection of HIV-1 antigens in spermatozoa after treatment with anti-HIV-1 polyclonal antibodies. (*a*) Gold particles (*arrow*) are localized on an electrodense particle in the cytoplasm around the sperm head of an HIV-1–infected patient. (*b*) Gold granules (*arrow*) are visible in the cytoplasm around the mitochondrial helix in a spermatozoon incubated in vitro with HIV-1. *A*, acrosome; *Ax*, axoneme; *M*, mitochondria; *N*, nucleus. Bars, 0.15 μm in *a* and 0.20 μm in *b*.

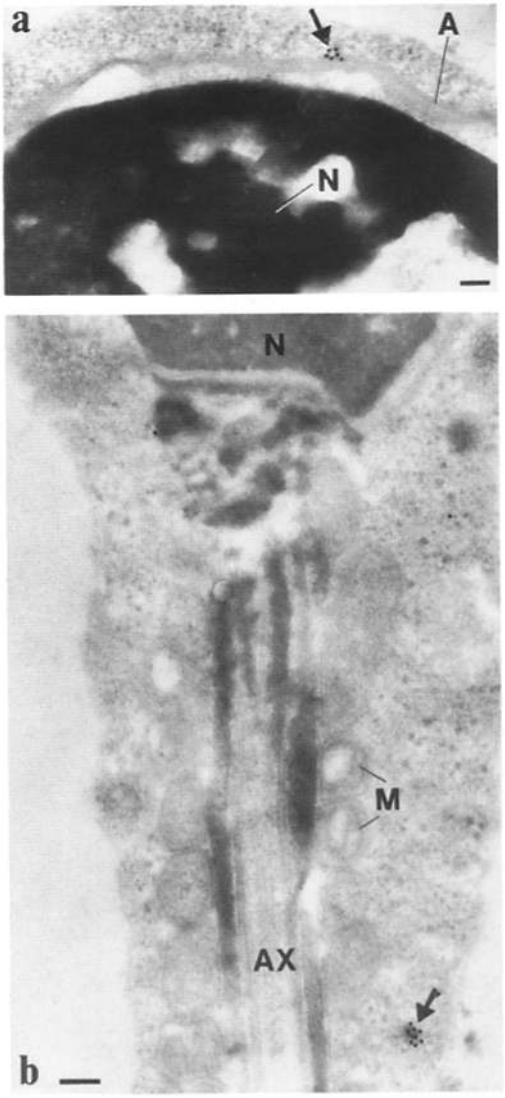


Figure 5. Immunogold detection of p24 antigen in spermatozoa. (*A*) Gold particles (*arrow*) are localized close to the outer acrosomal membrane in a spermatozoon infected in vitro with HIV-1. (*b*) Gold particles (*arrow*) are localized around the mitochondrial helix in a spermatozoon of an HIV-1–infected patient. *A*, acrosome; *Ax*, axoneme; *M*, mitochondria; *N*, nucleus. Bars, 0.10 μm in *a* and *b*.

fact, a very specific binding with anti-p24 antibodies (see later), exactly on them.

The three kinds of particles inside sperm were typically found between the plasma membrane and the outer acrosomal membrane in the sperm head (Fig. 1, *a–d*), or in the neck and mitochondrial districts (Figs. 1, *f–g*, and 2, *a–c*), regions where the space free from organelles is usually larger for the persistence of cytoplasmic residues. In some sections, they also appeared between the nucleus and the inner acrosomal membrane. In spermatozoa with reacting acrosomes, the particles are interspersed among many vesicles surrounding the nucleus, mostly derived from acrosomal membranes. Some of them have a diameter similar to the virus particles, but never show a nucleoid-like core. Spermatozoa from healthy donors (we have examined several hundreds of fertile and infertile men in many years) always appear free of virus-like particles.

In experiments carried out to analyze the time course of the viral binding and penetration, virus-like particles were only found attached to the outer surface of spermatozoa incubated with HIV-1 for 2 h at 4°C, and were washed before fixation (Fig. 2 *d*). If the incubation was continued for other 4 h at 37°C, virus-like particles, similar to those shown in Fig. 2 *d*, were found inside the sperm cytoplasm.

In sections of Lowicryl-embedded sperm from 8 out of the 15 infected patients (Figs. 4 *a* and 5 *b*) and of HIV-1–incubated sperm from the 15 healthy men (Figs. 4 *b* and 5 *a*), monoclonal anti-p24 antibody or polyclonal anti HIV-1 sera showed binding sites, appearing as clusters of 4–10 gold particles exclusively localized in the acrosomal (Figs. 4 *a* and 5 *a*) or mitochondrial regions (Fig. 4 *b* and 5 *b*). It is worth

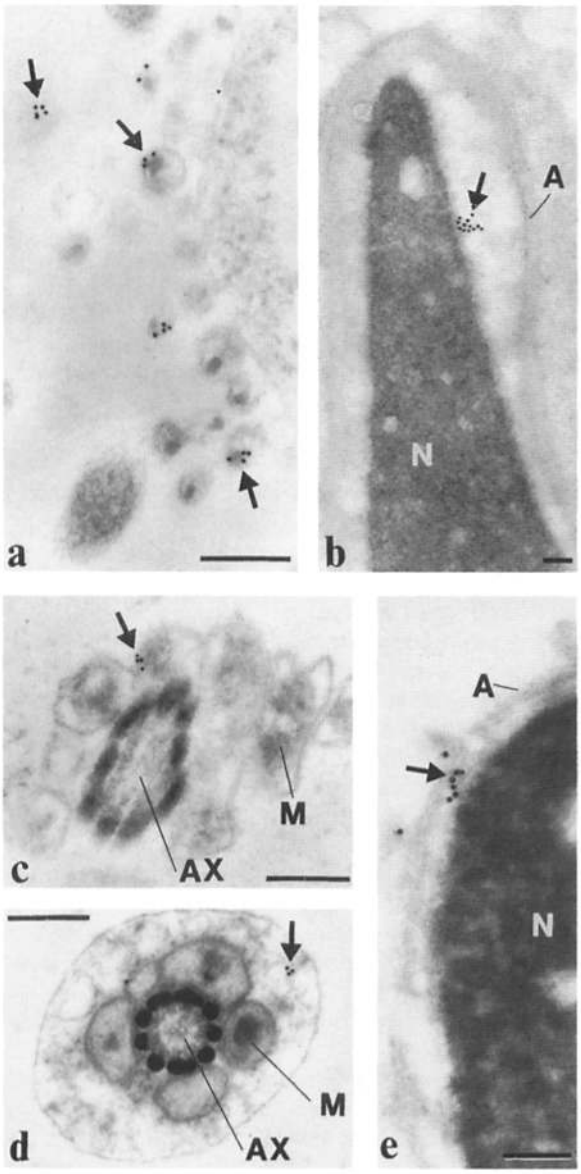


Figure 6. Immunogold labeling of HIV-1 RNA by in situ hybridization with a biotinylated DNA probe. (*a*) Detection of viral RNA (*arrows*) in the nucleoid of extracellular virions released from C8166 T infected cells. (*b* and *e*) Detection of viral RNA (*arrow*) in the perinuclear region in spermatozoa of HIV-1–infected patients (*b*) and HIV-1 incubated spermatozoa from seronegative men (*e*), respectively. (*c* and *d*) Detection of viral RNA (*arrow*) in the cytoplasmic area surrounding the mitochondrial helix respectively in spermatozoa of HIV-1–infected patients (*c*) and spermatozoa from seronegative men incubated in vitro with HIV-1 (*d*). *A*, acrosome; *Ax*, axoneme; *M*, mitochondria; *N*, nucleus. Bars, 0.20 μm in *a*, 0.10 μm in (*b*), 0.25 μm in *c* and *d*, and 0.15 μm in *e*.

stressing that the virus-like particles had always been found by conventional electron microscopy in the same districts. The gold particles are frequently localized exactly on moderately electron-dense structures having the same dimensions of the viral nucleoids described above (Figs. 4 *a* and 5 *b*). The lack of the viral envelope and the poor preservation of the nucleoid are probably caused by the slight fixation and to the kind of resin used for the embedding, which do not

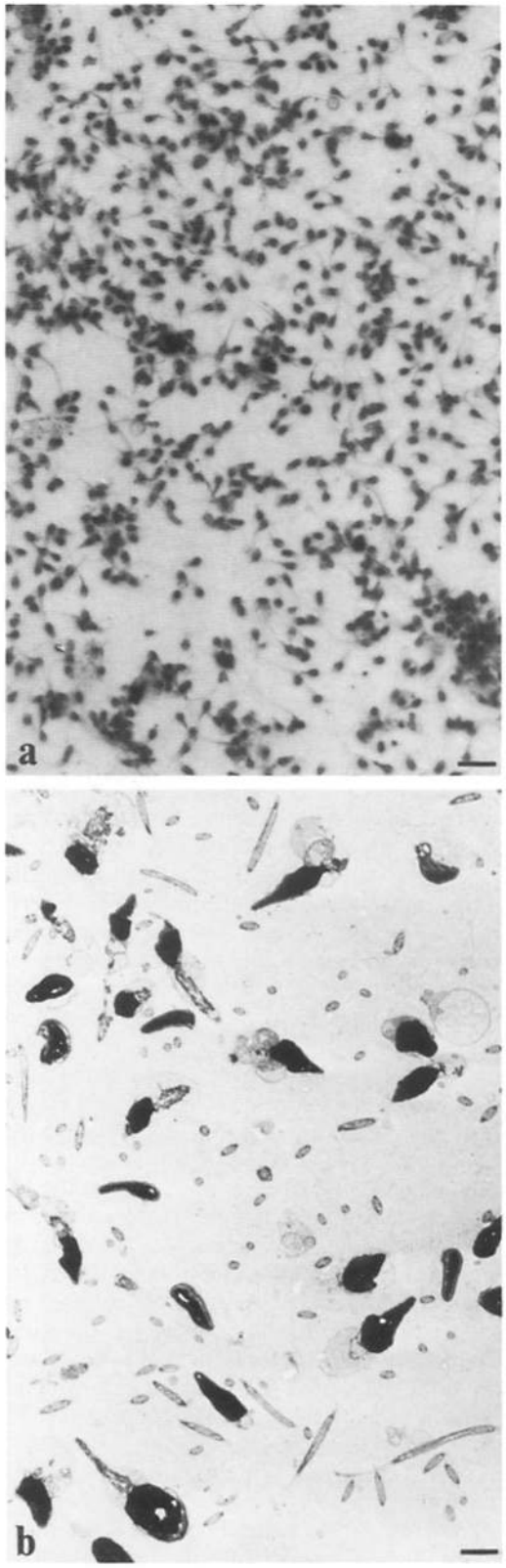


Figure 7. (*a*) Light micrograph of smeared spermatozoa from an HIV-1 infected patient after purification; only mature spermatozoa are present. (*b*) Electron micrograph of the same preparation, where only sections of sperm heads and tails are visible. *Bars*, 30 μm in *a* and 2 μm in *b*.

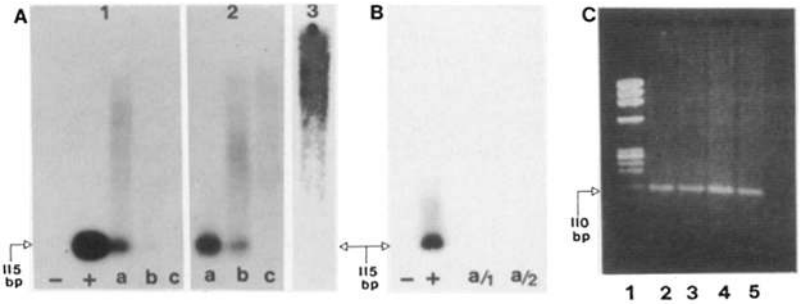


Figure 8. HIV-1 detection by PCR and RT-PCR in spermatozoa of two HIV-1–infected patients and of a healthy man as control. (*A1* and *A2*) HIV-1 RNA detection by RT-PCR in two HIV-1–infected patients: lane −, medium of uninfected C8166 T cell line; lane +, medium of C8166 T cell line 72 h after HIV-1 infection; lane *a*, pellet of purified spermatozoa fraction; lane *b*, pellet of Ficoll fraction; lane *c*, pellet of Ficoll supernatant. (*A3*) RT-PCR control for HIV-1 RNA of purified spermatozoa fraction obtained from a healthy donor. Time of filter exposure = 24 h. (*B*) HIV-1 DNA amplification by PCR: lane −, lysate of uninfected C8166 T cells (10^5 cells); lane +, lysate of 10^5 C8166 T cells 72 h after HIV-1 infection; lanes *a/1* and *a/2*, lysates of 10^6 purified spermatozoa from semen of N.1 and N.2 donors. Time of filter exposure = 8 h. (*C*) Ethidium bromide–stained gel of the PCR products amplified with a primer pair specific for beta-globin: lane *1*, size marker ϕX 174 (HaeIII digest); lane *2*, uninfected C8166; lane *3*, infected C8166; lane *4*, spermatozoa of N. 1 donor; lane *5*, spermatozoa of N. 2 donor.

allow a good morphological appearance. This picture is similar in HIV-1–infected C8166 T cells treated with the same technique (Fig. 3 *c*), where the high specificity of the binding is evident. Controls incubated only with the secondary antibody showed no binding, as did spermatozoa from healthy donors and uninfected C8166 T cells that were used as negative controls.

Detection of Sperm-associated HIV by In Situ Hybridization

To confirm the presence of HIV-1 in the sperm, in situ hybridization was carried out both on sperm from infected patients (Fig. 6, *b* and *c*) and on HIV-infected sperm from seronegative men (Fig. 6, *d* and *e*). Infected C8166 T cells served as positive controls (Fig. 6 *a*). Sections of Lowicryl-embedded material were incubated with the 9-kb biotin-labeled probe or with the mixture of biotinylated oligonucleotides representing sequences within the LTR, *gag*, and *env* regions of the viral genome. The sections were then washed and incubated with anti-biotin antibodies, followed by gold-conjugated anti IgG. Antibody binding is visible in the perinuclear region close to the acrosome (Fig. 6, *b* and *e*) and in the midpiece around mitochondria (Fig. 6, *c* and *d*) in the 15 in vitro–incubated samples and in 8 of the 15 infected patients, where the immunogold labeling was positive. The localization of the clusters of gold particles is the same as in the immunogold preparations (compare with Figs. 4 and 5) and in normal morphological preparations (Figs. 1 and 2). Similar binding occurred on HIV-1 particles in cultured infected C8166 T cells (Fig. 6 *a*). No binding was observed in control sections where the incubation with the probes was omitted and in the samples used as negative controls (uninfected spermatozoa and C8166 T cells).

Detection of Sperm-associated HIV-1 RNA by RT-PCR

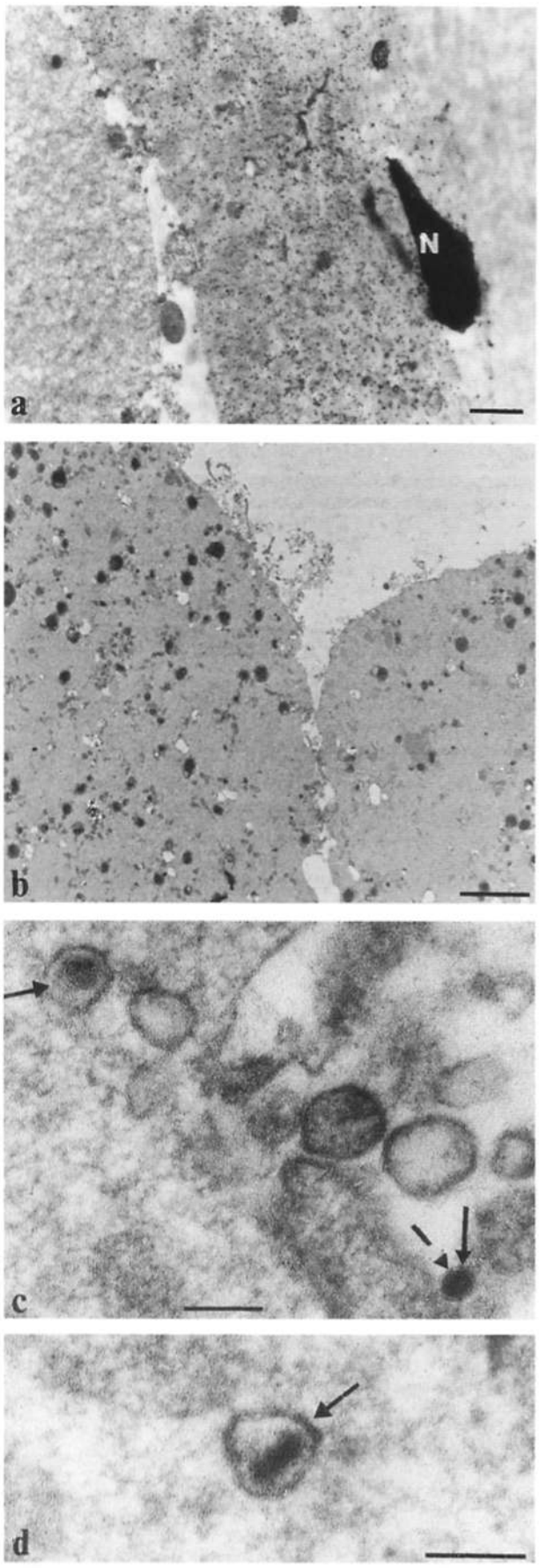
Spermatozoa prepared as pellet from Ficoll gradient (purified spermatozoa fraction) were examined by light and electron microscopy to confirm that leukocytes were absent (Fig. 7, *a* and *b*). In smeared material (Fig. 7 *a*) and in sections examined by electron microscopy (Fig. 7 *b*), only spermatozoa were seen. The ultrastructural characters of these cells were in the range of fertile men, according to the formulas of Baccetti and Mirolli (1994) and Baccetti et al. (1994*a* and 1994*b*). At higher magnifications, no virus particles were seen, either free or associated with the outer sperm surface.

RNA extracted from purified spermatozoa, Ficoll interface, and Ficoll supernatant fractions prepared from the sperm of two seropositive patients and from a healthy donor was reverse transcripted and then amplified with the SK38-39 primers as described in Materials and Methods. After two rounds of amplification, the spermatozoa and the Ficoll interface fractions from both patients showed positive signals, with the highest intensity in the spermatozoa fractions (Fig. 8, *A1* and *A2*, lanes *a* and *b*). The 115-bp band was absent from the spermatozoa obtained from the healthy donor (Fig. 8, *A3*). The supernatant fraction, which apparently did not contain spermatozoa, was PCR negative (Fig. 8, *A1* and *A2*, lanes *c*). The absence of contaminating DNA in our DNAse I–treated sperm extracts was demonstrated by the fact that the PCR, performed by specific primers for beta-globin gene, gave negative results (not shown). These experiments indicate that HIV RNA was present in the spermatozoa from the investigated patients.

The absence of proviral DNA in spermatozoa was further established by performing PCR analysis of lysates from Ficoll-purified spermatozoa, using *gag*-specific primers. As shown in Fig. 8 *B*, spermatozoa of both investigated patients (lanes *a/1* and *a/2*) were negative, whereas lysates from HIV-infected C8166 T cells (positive control) gave a strong positive signal (Fig. 8 *B*, lane +). The PCR of both spermatozoa samples was also still negative when the time of filter exposure was prolonged up to 72 h. All the cell lysates (purified spermatozoa and C8166 T cells) were positive in PCR performed by the primer pair PCO3 and PCO4, which amplifies a portion of beta-globin gene, indicating that lysis procedure we used successfully released DNA as template for PCR amplification (Fig. 8 *C*, lanes 2–5).

Transfer of HIV-1 by Fertilization

Purified motile sperm from infected donors added to culture dishes containing mature oocytes were observed to undergo morphologically normal acrosome reactions and to penetrate the zona pellucida (Fig. 9 *a*). After the fertilized eggs had reached the eight blastomere stage, they were fixed and embedded for transmission electron microscopy (Fig. 9 *b*). Fig. 9, *c–d*, shows that the cells of such embryos contain virus particles identical to those present in the sperm used for fertilization. In Fig. 9 *c*, representing the periphery of an



eight-blastomere embryo, a cross-sectioned particle with a 42-nm diameter circular nucleoid and an 84-nm diameter wall is shown; in the lower part of the same figure, a particle belonging to the third category described in "Electron Microscopic Identification of HIV-1 in Sperm" is seen, adhering to the outer surface of a blastomere. In Fig. 9 *d*, a longitudinally sectioned particle shows an elongated nucleoid measuring 70×27 nm contained in a vesicular wall with a 100-nm diameter. This kind of particle has never been found in normal fertilized oocytes at the same stage.

Alternative HIV Receptors in the Plasma Membrane of Spermatozoa

Antigalactosylceramide antibodies to bind to the midpiece and the equatorial segment of all the living or fixed human spermatozoa (Fig. 10, *a–d*). No binding was seen in control experiments when an uncorrelated isotype was used (Fig. 10, *e–f*) or the primary antibody was omitted (Fig. 10, *g* and *h*). Specificity of the antibodies (Sommer and Schachner, 1981; Bensal et al., 1989) has been reexamined under our experimental conditions using cells known to express the antigen (mouse Schwann cells) (Fig. 11, *a* and *c*) or known as being devoid of it (Swiss 3T3 cells) (Fig. 11, *b* and *d*).

Discussion

In this research, we performed electron microscopy and immunoelectron microscopy study on sperm from HIV-1–infected donors and on seronegative donor sperm incubated with HIV-1 in vitro. By these methods, we have shown that virus particles containing HIV-1 antigens can penetrate in human spermatozoa in vitro and in vivo.

We believe that these particles represent HIV-1 virions for the following reasons: (*a*) The particles found both in AIDS patients sperm and in sperm from healthy donors in vitro infected (HIV-1/H9 stock and cocultivation with infected C8166 T cells) exhibit the same morphological features both in spermatozoa and in the stock of the infected cells. (*b*) The particles show the same immunocytochemical characters in the two types of spermatozoa and in the C8166 T cells, where the antibody binding is extremely specific. (*c*) In uninfected sperm used as controls and in spermatozoa of several hundreds healthy or sterile men examined by us until now, this kind of particle has never been detected.

These particles are in higher number in the healthy sperm infected in vitro. Both in infected patients and in in vitro–infected sperm from healthy donors, the structure and motility of the spermatozoa appear almost normal. We further

Figure 9. Sections of an oocyte fertilized in vitro with spermatozoa of an HIV-1–infected patient. (*a*) A sperm head with reacted acrosome is visible in the zona pellucida. (*b*) Section of two blastomeres of an eight-blastomere human preembryo after in vitro fertilization with spermatozoa of an HIV-1–infected patient. (*c* and *d*) HIV-1 particles (*arrows*) in the cytoplasm of the same blastomeres of (*b*). The electrondense nucleoid and the membrane-like coat are clearly visible. At the bottom of *d*, a particle devoid of membrane envelope is indicated (*double arrows*). *N*, sperm nucleus. Bars, 1 μm in *a*, 3 μm in *b*, and 0.10 μm in *c* and *d*.

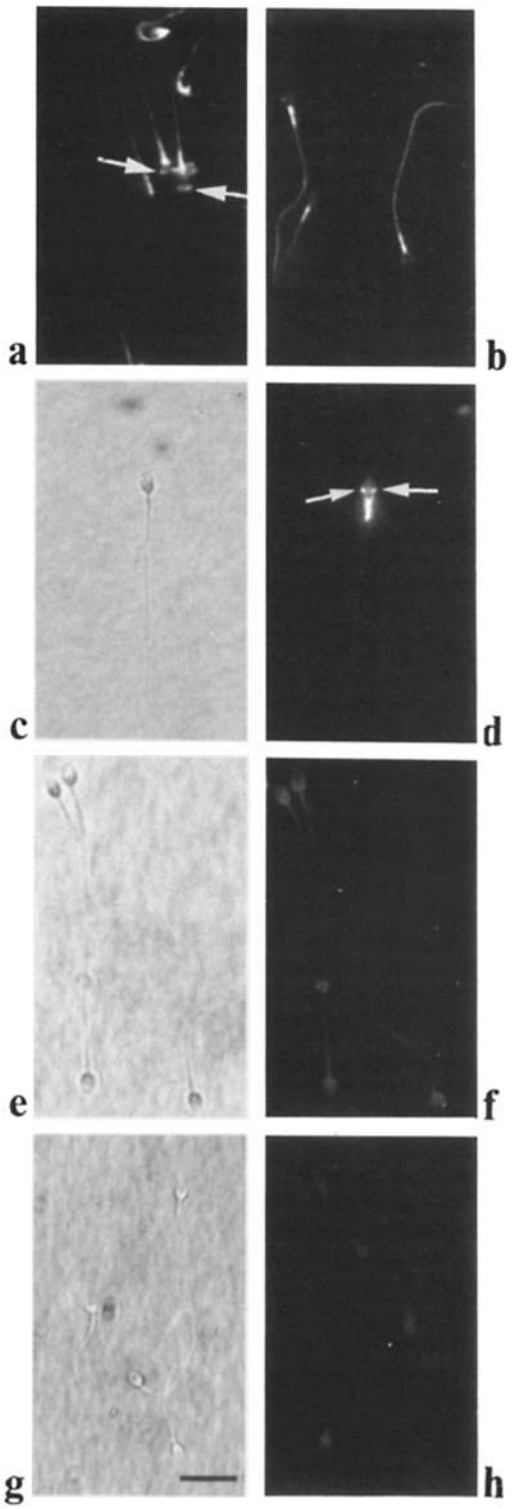


Figure 10. Immunofluorescence localization of mAb 01 (antigalactosylceramide) on human spermatozoa. (*a*) In prefixed sperm cells, the fluorescence is localized on the anterior portion of the middle piece, and the equatorial segment is also occasionally stained (*arrows*). (*b* and *d*) In spermatozoa treated with mAb 01 before fixation, the same localization as in *a* is visible. (*c*) Light microscopy image of *d*. (*e* and *f*) Light and immunofluorescence micrographs of spermatozoa treated with anti-IL-1 isotype control antibody. (*g* and *h*) Light and immunofluorescence micrographs of spermatozoa treated with only FITC-IgM second antibody. Bar, 10 μm.

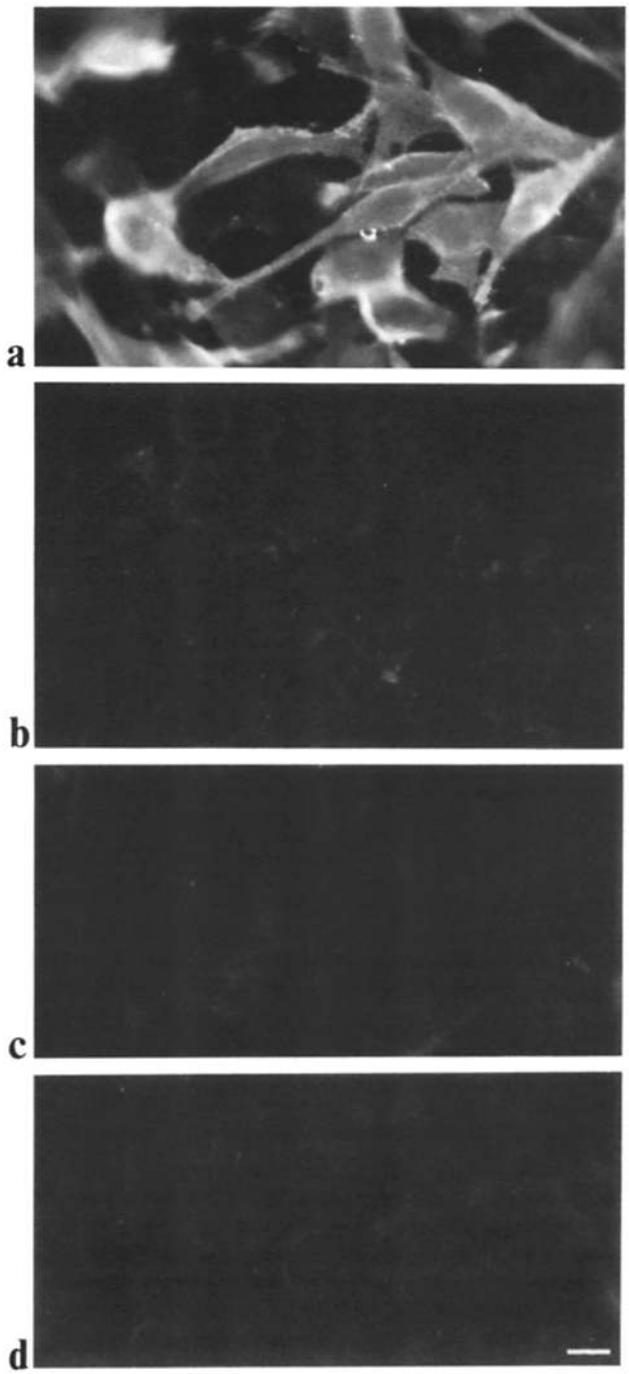


Figure 11. Immunofluorescence detection of mAb 01 on (*a*) Schwann cells clonal cell line A 6 and (*b*) Swiss 3T3 cells. (*c* and *d*) Schwann cells and Swiss 3T3 cells were incubated only with FITC-IgM secondary antibody. Bar, 10 μm.

corroborated these results using in situ hybridization and polymerase chain reaction.

Mermin et al. (1991) and Van Voorhis et al. (1991) failed to detect HIV-1 DNA in the sperm fraction of the semen of infected patients, while Scofield et al. (1992) identified such sequences in sperm prepared using methods that prevent lipid peroxidation. Our results disagree somewhat with those of Scofield and colleagues in that although viral RNA was present, denoting the presence of virus particles, we failed

to detect HIV-1 DNA in sperm. We agree with the suggestion by Scofield (1992) that virus particles in the sperm cytoplasm represent infecting, but not replicating, virions.

The HIV-1 particles in in vitro–infected sperm appear morphologically similar to those that appear in the sperm of AIDS patients. The most common particle morphology seen both in in vitro–infected sperm and in patients' sperm is that of nucleoids surrounded by a membrane envelope, which is typical of mature viruses. A second category is that of envelopes devoid of nucleoids and homogeneously full of granular material (atypical virus-like particles according to Tenner-Racz et al. [1988], who studied lymph nodes; immature virions according to Phillips and Tan [1992], who studied trophoblast cells). A third category in both kinds of spermatozoa is that of particles resembling bare nucleoids without envelopes. In the first case, intact virions may have penetrated via endocytosis (Maddon et al., 1986; Tateno et al., 1989; Grewe et al., 1990; Phillips and Tan, 1992), followed by fusion between viral and endosomal membranes (Pauza and Price, 1988). Also in the second case, virions may have entered via endocytosis. If the budding-like images observed in patient's sperm are well interpreted, this presence suggests the possibility that HIV virions could be also released from the spermatozoa. The last hypothesis is improbable because proviral DNA is usually absent in these spermatozoa (see below). In the third case (bare nucleoids), virions may have entered via membrane fusion (Goto et al., 1988; Stein et al., 1987; Stevenson et al., 1992).

We believe that the mature virus-like particles observed in our sperm samples represent virions penetrated via endocytosis, their uncoating being delayed or prevented by a low hydrolytic activity in the cytosol, as suggested by the absence of lysosomes in spermatozoa. The hypothesis that the virus particles seen in spermatozoa represent penetrating virions agrees with the failure to detect proviral DNA in the sperm fraction.

Recent reports (Lori et al., 1992; Trono, 1992), demonstrated that virions in purified stocks from infected cell lines contain HIV RNA associated to complementary copies of DNA. These data could be in contrast with the absence of HIV DNA in spermatozoa of our infected patients. This contrast, however, could be overcome assuming that the occurrence of DNA-containing virions is a rare event in vivo, as demonstrated by the absence of HIV-DNA in virions present in human plasma (Piatak et al., 1993).

Regardless of the molecular state of sperm-associated HIV, sperm may function as a vehicle for transfer of "dormant" virus to somatic cells or to eggs. For mucosal cells, infection may occur after sperm binding to HLA-DR on the cell surface (Ashida and Scofield, 1987; Miller and Scofield, 1990; Scofield et al., 1992*a*). For ovulated eggs, viral entry may occur either directly, via the fertilizing sperm, or through entry into the egg or zygote by virus particles carried by other sperm surrounding the egg and released during the acrosomal reaction.

The data presented here show that HIV-1 particles bind and enter normal human sperm, that sperm from HIV-seropositive men contain antigenically identifiable HIV-1 particles and nucleic acid, that such sperm are morphologically normal, motile and can fertilize oocytes and transfer virus particles to the resulting embryos. Detection of viral RNA by RT-PCR has medical significance for semen processing technologies now being employed for male-seropositive HIV disparate couples (Semprini et al., 1992). The latter study produced no seroconversions in 29 women inseminated with "washed" sperm, nor seropositive children. As a consequence the Authors concluded that spermatozoa don't contain HIV-1 particles. The present data suggest that the absence of HIV-1 in the women was probably due to the limited exposure of them to infected sperm (one insemination per superovulation cycle) rather than to the absence of HIV in the sperm preparations themselves.

The modality of penetration of HIV in the spermatozoa is still unclear due to the contradictory results obtained in the attempt to demonstrate the presence of CD4 on the sperm membranes (Bagasra et al., 1990; Gobert et al., 1990; Anderson, 1992). Ashida and Scofield (1987) and Scofield et al. (1992a), using a different approach, demonstrated that sperm can bind HIV through the mechanism related to class II MHC-CD4 molecules.

The data reported in this paper show that galactosylceramide or a related compound is usually present on the surface membrane of human spermatozoa and could function as an alternative receptor to HIV as proposed by Bath et al. (1991) and by Harouse et al. (1991) for neural cells, and by Fantini et al. (1993) and Yahi et al. (1992) for epithelial colon cell lines. Identification studies of the molecules involved in the binding of antigalactosylceramides are under way in our laboratory.

These findings substantially improve our knowledge of the interaction between HIV-1 and sperm, but they also raise new questions. Can HIV replicate in sperm, or is it merely stored and carried by these cells? Do sperm acquire HIV during spermatogenesis or after release into the epididymis? What is the fate of virus particles transferred into oocytes? Work on these issues is in progress.

8E5/LAV cells were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute for Acquired Immune Deficiency, National Institutes of Health from Dr. Thomas Folks.

This research was supported by Istituto Superiore Sanità, AIDS Research Project 1993, and by the National Research Council of Italy, Special Project FATMA, subproject 5.

Received for publication 30 March 1993 and in revised form 22 August 1994.

References

Anderson, D. J. 1992. Mechanisms of HIV-1 transmission via semen. *J. NIH Res.* 4:104 and 106–108.

Anderson, D. J., H. Wolff, J. Pudney, Z. Whenhao, A. Martinez, and K. Mayer. 1990. Presence of HIV in semen. *In* Heterosexual transmission of AIDS. N. J. Alexander, H. L. Gebelnick, and J. M. Spieler, editors. Wiley-Liss, Inc., New York. pp. 167–180.

Ashida, R. A., and V. L. Scofield. 1987. Lymphocyte major histocompatibility complex-coded class II structures may act as sperm receptors. *Proc. Natl. Acad. Sci. USA.* 84:3395–3399.

Baccetti, B., A. Benedetto, A. G. Burrini, G. Collodel, G. Elia, P. Piomboni, T. Renieri, and M. Zaccarelli. 1990. Retrovirus-like particles detected in spermatozoa of patients with AIDS. *Int. Symp. on AIDS and Reproduction, Genoa.* 4 (*Abstr.*)

Baccetti, B., A. Benedetto, A. G. Burrini, G. Collodel, G. Elia, P. Piomboni, T. Renieri, C. Sensini, and M. Zaccarelli. 1991. HIV particles detected in spermatozoa of patients with AIDS. *J. Submicr. Cytol. Pathol.* 23:339–345.

Baccetti, B., A. Benedetto, A. G. Burrini, G. Collodel, G. Elia, P. Piomboni, T. Renieri, and M. Zaccarelli. 1992. Spermatozoa of patients with AIDS contain HIV particles. *In* AIDS and Human Reproduction. F. Mélica, editor. Karger, Basel. pp. 47–54.

Baccetti, B., G. Bernieri, A. G. Burrini, G. Collodel, N. Crisà, M. Mirolli, E. Moretti, and P. Piomboni. 1994*a*. Notulae seminologicae. 5. Mathematical evaluation of interdependent submicroscopic sperm alterations. *J. An-*

drol. In press.
Baccetti, B., A. G. Burrini, G. Collodel, M. Mirolli, E. Moretti, and P. Piomboni. 1994*b*. Seminological investigations by submicroscopical methods. *In* Perspective on Assisted Reproduction. T. Mori, T. Tominaga, T. Aono, and M. Hiroi, editors. *Front. Endocrinol.* 4:609–615.
Baccetti, B., and M. Mirolli. 1994. Notulae seminologicae. 3. Mathematical diagnosis from TEM seminological detection. *Andrologia.* 26:47–49.
Bagasra, O., M. Freund, J. Weidmann, and G. Harley. 1988. Interaction of human immunodeficiency virus with human sperm in vitro. *J. Acq. Immun. Syndr.* 1:431–435.
Bagasra, O. and M. Freund. 1990. In vivo and in vitro studies of HIV-1 and human sperm. In "Heterosexual transmission of AIDS". N. J. Alexander, H. L. Gebelnick, and J. M. Spieler, editors. Wiley-Liss, New York. pp. 155–166.
Bagasra, O., M. Freund, D. Condoluci, B. Heins, P. Whittle, J. Weidmann, and J. Comito. 1990. Presence of HIV-1 in sperm of patients with HIV/AIDS. *Mol. Androl.* 2:109–125.
Bath, S., S. L. Spitalnik, F. Gonzalez-Scarano, and D. H. Silberberg. 1991. Galactosylceramide or a derivative is an essential component of the neural receptor for human immunodeficiency virus type 1 envelope glycoprotein gp120. *Proc. Natl. Acad. Sci. (USA).* 88:7131–7134.
Benedetto, A., A. R. Garbuglia, A. Di Caro, E. Lo Presti, E. Alfani, and C. Delfini. 1993. Virus-free survival and down-regulation of CD4 in C8166 cells infected with human immunodeficiency virus type 1 at low density. *J. Gen. Virol.* 74:2595–2601.
Bensal, R., A. E. Warrington, A. L. Gard, B. Rauscht, and S. E. Pfeiffer. 1989. Multiple and novel specificities of monoclonal antibodies 01, 04 and R-mAb used in the analysis of oligodendrocyte development. *J. Neurosci. Res.* 24: 548–557.
Carlemalm, E., W. Villiger, J. A. Hobot, J. D. Acetarin, and E. Kellenberger. 1985. Low temperature embedding with Lowicryl resins: two new formulations and some applications. *J. Microsc.* 140:55–63.
Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidium thyocianate-phenol-chloroform extraction. *Anal. Biochem.* 162:156–159.
Fantini, J., D. G. Cook, N. Nathanson, S. Spitalnik, and F. Gonzales-Scarano. 1993. Infection of colonic epithelial cell lines by type 1 human immunodeficiency virus is associated with cell surface expression of galactosylceramide, a potential alternative gp120 receptor. *Proc. Natl. Acad. Sci. USA.* 90:2700–2704.
Folks, T. M., D. Powell, M. Lightfoote, S. Koenig, A. S. Fauci, S. Benn, A. Rabson, D. Daugherty, H. E. Gendelman, M. D. Hoggan, S. Venkatesan, and M. A. Martin. 1986. Biological and biochemical characterization of a cloned Leu-3$^-$ cell surviving infection with the acquired immune deficiency syndrome retrovirus. *J. Exp. Med.* 164:280–290.
Fournier, J. G., F. Escaig-Haye, and V. Grigoriev. 1991. Visualization of ribonucleic acid sequences after in situ hybridization with non-isotopic probes and immuno-gold electron microscopy. *Boll. Soc. Ital. Microsc. Elettr.* 2: 104–114.
Gobert, B., C. Amiel, J. Tang, P. Barbarino, M. C. Béné, and G. Faure. 1990. CD4-like molecules in human sperm. *FEBS (Fed. Eur. Biochem. Soc.) Letters.* 261:339–342.
Goto, T., S. Harada, N. Yamamoto, and M. Nakai. 1988. Entry of human immunodeficiency virus (HIV) into MT-2, human T cell leukemia virus carrier cell line. *Arch Virol.* 102:29–38.
Grewe, C., A. Beck, and H. R. Gelderblom. 1990. HIV: early virus-cell interactions. *J. Acq. Immun. Syndr.* 3:965–974.
Harouse, J. M., S. Bhat, S. L. Spitalnik, M. Laughlin, K. Stefano, D. K. Silberberg, and F. Gonzales-Scarano. 1991. Inhibition of entry of HIV-1 in neural cell lines by antibodies against galactosyl ceramide. *Science (Wash. DC).* 253:320–323.
Lori, F., F. Di Marzo Veronese, A. L. De Vico, P. Lusso, M. S. Reitz, Jr., and R. C. Gallo. 1992. Viral DNA carried by human immunodeficiency virus type 1 virions. *J. Virol.* 66:5067–5074.
Maddon, P. J., A. G. Dalgleish, J. S. McDougal, P. R. Clapman, R. A. Weiss, and L. Axel. 1986. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. *Cell.* 47:333–348.
Mermin, J. H., M. Holodniy, D. A. Katzenstein, and T. C. Merigan. 1991. Detection of human immunodeficiency virus DNA and RNA in semen by the polymerase chain reaction. *J. Infect. Dis.* 164:769–772.
Miller, V. E., and V. L. Scofield. 1990. Transfer of HIV by semen: role of sperm: *In* Heterosexual transmission of AIDS. N. J. Alexander, H. L. Gebelnick, and J. M. Spieler, editors. Wiley-Liss, Inc., New York. pp. 147–154.
Ou, C. Y., S. Kwok, S. W. Mitchell, D. H. Mack, J. J. Sninsky, J. W. Krebs, P. Feorinzo, D. Warfield, and G. Schochetman. 1988. DNA amplification for direct detection of HIV-1 in DNA of peripheral blood mononuclear cells. *Science (Wash. DC).* 239:295–297.
Pauza, C., and T. M. Price. 1988. HIV infection of T cells and monocytes proceeds via receptor-mediated endocytosis. *J. Cell Biol.* 107:959–968.
Phillips, D. M., and X. Tan. 1992. HIV-1 infection of the trophoblast cell line BeWo: a study of virus uptake. AIDS Res. and Human Retrovirus. 8: 1683–1691.
Piatak, M., Jr., M. S. Saag, L. C. Yang, S. J. Clark, J. C. Kappes, K. C. Luk, B. H. Hahn, G. M. Shaw, and J. D. Lifson. 1993. High levels of HIV-1 in plasma during all stages of infection determined by competitive PCR. *Science (Wash. DC).* 259:1749–1754.
Pudney, J. 1990. Caveats associated with identifying HIV using transmission electron microscopy. *In* Heterosexual transmission of AIDS. N. J. Alexander, H. L. Gelbelnick, and J. M. Spieler, editors. Wiley-Liss, Inc., New York. pp. 197–204.
Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty percent endpoints. *Am. J. Hyg.* 27:493–497.
Saiki, R. K., T. L. Bugawon, G. T. Horn, K. B. Mullis, and H. A. Erlich. 1986. Analysis of enzymatically amplified beta-globin and HLA-DQa DNA with allele-specific oligonucleotide probes. *Nature (Lond.)* 324:163–166.
Scofield, V. L. 1992. Sperm as vectors and cofactors for HIV-1 transmission. *J. NIH Res.* 4:105 and 108–111.
Scofield, V. L., R. Clisham, L. Bandyopadhyay, P. Gladstone, L. Zamboni, and R. Raghupathy. 1992*a*. Binding of sperm to somatic cells via HLA-DR: modulation by sulfated carbohydrates. *J. Immunol.* 148:1718–1724.
Scofield, V. L., B. Poiesz, C. Kennedy, S. Broder, A. Diagne, and B. Rao. 1992*b*. HIV binds to normal sperm and is present in sperm from infected men. *Int. Conf. on AIDS*, VIII. *Amsterdam.* 2102 (*Abstr.*).
Semprini, A. E., P. Levi-Setti, M. Bozzo, M. Ravizza, A. Taglioretti, P. Sulpizio, E. Albani, M. Oneta, and G. Pardi. 1992. Insemination of HIV-negative women with processed semen of HIV-positive partners. *Lancet* 340:1317–1319.
Sommer, I., and M. Schachner. 1981. Monoclonal antibodies (01 to 04) to oligodendrocyte surfaces. An immunocytochemical study in the central nervous system. *Dev. Biol.* 83:311–317.
Stein, B. S., S. Gowda, J. Lifson, R. Penhallow, K. Bensch, and E. Engleman. 1987. pH-independent HIV entry into CD4-positive T cells via virus envelope fusion to the plasma membrane. *Cell.* 49:659–668.
Stevenson, M., M. Bukrinsky, and S. Haggerty. 1992. HIV-1 replication and potential targets for intervention. *AIDS Res. Hum. Retrov.* 8:107–117.
Tateno, M., F. Gonzalez-Scarano, and J. A. Levy. 1989. Human immunodeficiency virus can infect CD4-negative human fibroblastoid cells. *Proc. Natl. Acad. Sci. USA.* 86:4287–4294.
Tenner-Racz, K., P. Racz, S. Gartner, M. Dietrich, and M. Popovic. 1988. Atypical virus particles in HIV-1-associated persistent generalised lymphadenopathy. *Lancet.* 2:774–775.
Trono, D. 1992. Partial reverse transcripts in virions from immunodeficiency and murine leukemia viruses. *J. Virol.* 66:4893–4900.
Van Voorhis, B. D., A. Martinez, K. Mayer, and D. J. Anderson. 1991. Detection of HIV-1 in semen from seropositive men using culture and polymerase chain reaction deoxyribonucleic acid amplification techniques. *Fertil. Steril.* 55:588–594.
Wolff, H., W. H. Zhang, and D. J. Anderson. 1988. The CD4 antigen (HIV-receptor) is not detectable on human testicular germ cells or spermatozoa. *Intern. Conf. on AIDS, IV. Stockholm.* 2547:112 (*Abstr.*).
Yahi, N., S. Baghdiguian, H. Moreau, and J. Fantini. 1992. Galactosyl ceramide (or a closely related molecule) is the receptor for human immunodeficiency virus type 1 on human colon epithelial HT29 cells. *J. Virol.* 66:4848–4854.