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Sperm-associated retroviruses in the mouse epididymis

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ABSTRACT **Sperm adsorbed with retrovirus particles were recovered from the epididymis of apparently normal male mice. Epididymal semen from all four mouse strains examined was positive for retrovirus (10^5 to 10^8 particles per μg of protein) indicating that epididymal fluids and sperm may be important vehicles for murine retrovirus spread. Immunoblot analyses revealed that the banding patterns of electrophoretically separated epididymal viral proteins from the four strains of males were more similar to each other than to either xenotropic New Zealand Black virus or ecotropic Rauscher leukemia virus proteins. The results indicate that retrovirus particles, possibly a unique strain, are commonly expressed at relatively high titers in the reproductive tract of male mice and are sperm-associated.**

A complete understanding of the transmissibility of retroviruses requires understanding the interaction of retroviruses with reproductive tract and embryonic cells. Although a principal mode of transmission of acquired immune deficiency syndrome is through sexual contact with seminal fluid that contains human immunodeficiency virus (1–6; §), retrovirus infection of the human reproductive tract is not understood nor is it known if other retroviruses, animal or human, are spread through sexual contact with reproductive tract secretions. The detection of retroviral reverse transcriptase activity in cell-free epididymal fluids from several strains of mice (7) and reports of viral glycoprotein, gp70, expression in mouse epididymis (8) indicated that retrovirus particles may be common in the reproductive tract of male mice. Based on the amount of reverse transcriptase activity detected, virus concentration estimates were as high as 10^9 particles per μl of epididymal semen (7, 9). Epididymal fluids could thus be an important vehicle for murine retrovirus spread. In addition, partially purified sperm retained some of the reverse transcriptase activity, implying an association between the sperm cells and the reproductive tract retroviruses. Sperm-bound retroviruses would have access to all the cells contacted by motile sperm, including oocytes.

To begin to determine if murine retroviruses could be spread by sexual contact, we have examined epididymal semen from four mouse strains: two known to contain endogenous xenotropic retroviral gene sequences, New Zealand Black (NZB) and random-bred Swiss (10), and two known to contain endogenous ecotropic as well as xenotropic retroviral gene sequences, C57BL/6 and AKR (11). The results reported here indicate that retrovirus particles are common in mouse epididymal semen and are sperm-associated.

MATERIALS AND METHODS

Mouse Strains and Epididymal Semen Preparations. Mature males, 6–12 weeks of age, or retired breeders were from VAF (virus-antibody free) stocks. Strains C57BL/6, AKR, and New Zealand Black (NZB) were from the Jackson Laboratories; Cox Swiss males were from Laboratory Supply (Indianapolis, IN). Semen from the cauda epididymis and ductus deferens was collected in buffer A [0.01 M Tris·HCl (pH 7.4), 0.1 M KCl, bovine serum albumin at 1 mg/ml (BSA; Fraction V, Sigma)] with the protease inhibitors leupeptin at 100 μg/ml and soybean trypsin inhibitor at 100 μg/ml (Sigma) and assayed for protein as described (7). Epididymal samples were from pools of at least three males per strain and were obtained in most cases within 2–7 days of shipment.

Reference Viruses and Antibodies. Xenotropic NZB virus (C135), ecotropic Rauscher leukemia virus (RLV, strain JLSV9), and primary antibodies anti-RLV p30 (goat) and anti-RLV gp70 (goat) were from the Biological Carcinogenesis Branch, National Cancer Institute; anti-NZB gp70 antibody (goat) was also a gift from J. Elder (12); control goat serum was from Sigma.

Immuno Dot Blots. Reference viruses and antigens were dotted onto washed nitrocellulose filters as 2-μl samples of serial 1:2 dilutions, and nonspecific binding sites on filters were blocked with Blotto [5% (wt/vol) nonfat dry milk (Carnation), 0.01% (vol/vol) Antifoam B (Sigma), 0.001% (wt/vol) Thimerosol (Sigma), in PBS] (13) for 1 hr and washed three times with PBS (140 mM NaCl/3 mM KCl/8 mM Na_2HPO_4/1.5 mM KH_2PO_4, pH 7.2) containing BSA at 1 mg/ml. Primary antibodies were diluted 1:500, preabsorbed with reproductive tract antigens (0.5 mg of C57BL/6 testes homogenate and 10^5 twice-washed C57BL/6 sperm per ml of diluted antibody) for 45 min, and centrifugation for 60 min at 100,000 × g. Filters were incubated with primary antibody for 1 hr at room temperature and then rinsed three times with PBS/BSA. Secondary antibody (biotinylated donkey anti-goat IgG, Bethesda Research Laboratories) was diluted 1:1000 and preabsorbed with 3 mg of NZB testis homogenate, 2.6 mg of epididymis homogenate, and 2 × 10^5 C57BL/6 twice-washed sperm per ml of diluted antibody for 45 min at 4°C followed by centrifugation at 100,000 × g for 60 min at 4°C. Dot blots were incubated with secondary antibody for 60 min at room temperature followed by three rinses with PBS/BSA. Streptavidin-conjugated β-galactosidase (Bethesda Research Laboratories), diluted 1:500 in the PBS/BSA, was allowed to react with the dot-blot-bound biotinylated secondary antibody for 30 min. Following three rinses with PBS/BSA, the dot blots were developed in a solution of β-galactosidase substrate (Bluo-gal, Bethesda Research Laboratories) at 0.43 mg/ml in 3.1 mM $K_3Fe(CN)_6$/3.1 mM $K_4Fe(CN)_6$/10 mM sodium phosphate, pH 7.2/0.15 M NaCl/1.0 mM $MgCl_2$ for 1 hr. Dot blots incubated with control, preabsorbed goat serum as primary antibody were routinely negative.

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Abbreviations: BSA, bovine serum albumin; RLV, Rauscher leukemia virus; NZB, New Zealand Black.

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§Borzy, M. & Kiessling, A. A., International Conference on Acquired Immune Deficiency Syndrome, April 14–17, 1985, Atlanta, p. 51 (abstr.).

Immunoblots. Epididymal antigens were denatured in a solution of 0.06 M Tris·HCl (pH 6.8), 2% (wt/vol) $NaDodSO_4$, 10% (vol/vol) glycerol, and 5% (vol/vol) 2-mercaptoethanol. Electrophoresis was carried out in 10% polyacrylamide gels for 45–60 min at 25 mA per gel (8). Proteins were transferred to nitrocellulose at high voltage in 0.025 M Tris/0.192 M glycine, pH 8.3, containing 20% (vol/vol) methanol. Seroreactive protein bands were identified as described for immuno dot blots.

Electron Microscopy. Cauda epididymides were removed from healthy males and immediately fixed in PBS containing 2% (vol/vol) glutaraldehyde and 1% paraformaldehyde. Tissues were stained with osmium tetroxide prior to embedding in Epon. Sections, 800 Å, were poststained with 0.08 M lead citrate/saturated uranyl acetate prior to viewing.

Fluorescence Microscopy. Epididymal fluids were collected as described above, layered over a cushion of BSA (20 mg/ml) in buffer A with the protease inhibitors (as above), and centrifuged at 500 × *g* for 10 min to pellet the sperm. Sperm pellets were resuspended and air-dried on glass slides for indirect immunofluorescence of viral proteins. Incubations with blocking agents and primary and secondary antibodies were as described for dot blots. Antibody binding was visualized by the fluorescence of bound streptavidin-conjugated Texas Red (Bethesda Research Laboratories) bound to the biotinylated secondary antibody.

RESULTS

Epididymal Proteins Seroreactive with Antibodies Against Viral Proteins. Immuno dot blot analyses of epididymal fluids from the four mouse strains were positive for proteins seroreactive with antibody against retrovirus *gag* gene product, p30, and *env* gene product, gp70 (Fig. 1). Serial dilutions of two reference viruses, RLV and NZB virus, were compared with the serial dilutions of the epididymal fluids to provide a relative estimate of the virus particle equivalents of seroreactive antigen per μg of epididymal protein (Fig. 1). The virus particle equivalents of seroreactive epididymal proteins ranged from 10^7 to 10^8 virus particles per μg of protein for NZB males to from 10^5 to 10^6 virus particles per μg of protein for the C57BL/6 males, in agreement with reverse transcriptase assays of the same preparations (Table 1) and with reported results (7). The detection of virus was consistent for several groups of males received over a period of 15 months indicating the viral expression was not due to exogenous infection.

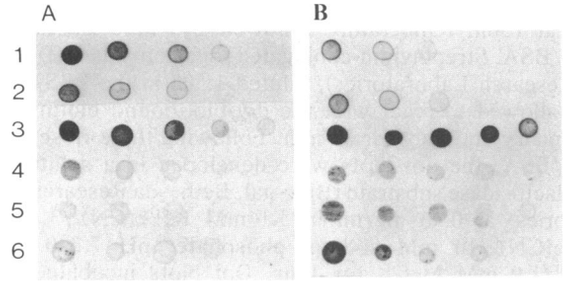

FIG. 1. Serial dilutions of retroviruses and epididymal fluids immuno dot blotted with anti-gp70 and anti-p30 antibodies. Semen from the cauda epididymis and ductus deferens of NZB, AKR, C57BL/6, and Swiss males was collected, and seroreactive viral proteins were detected. The rows of dots are 1:2 serial dilutions of the initial antigen concentrations. Rows: 1, NZB virus (1.2×10^7 virus particles); 2, RLV (1.4×10^7 virus particles); 3–6, epididymal proteins from NZB (1.4 μg), AKR (4 μg), C57BL/6 (4 μg), and Swiss (4 μg), respectively. (*A*) Anti-p30. (*B*) Anti-gp70.

Table 1. Estimated virus particle equivalents of virus protein in epididymal semen

Strain	Virus particles, no. per μg of protein
NZB	10^7–10^8
AKR	10^6–10^7
Swiss	10^6–10^7
C57BL/6	10^5–10^6

Values are estimates of the number of virus particles per μg of epididymal protein based on the results of the immuno dot blot titrations for gp70 and p30 (Fig. 1) and reverse transcriptase activity (7, 9) in each of the epididymal fluid samples used for immuno dot blot dilutions.

Immunoblot analyses indicated that the seroreactive proteins in the epididymal fluids corresponded in some, but not all, respects to seroreactive proteins in RLV and NZB virus analyzed simultaneously (Fig. 2). A distinct epididymal p30 band that comigrated with the viral p30 proteins was detected in all four mouse strains (Fig. 2*A*). The anti-p30 antibody reacted weakly with other viral proteins showing banding patterns of seroreactive epididymal proteins that were more similar to each other than to the RLV or the NZB viruses. The banding patterns were confirmed in parallel blots incubated with anti-gp70 antiserum (Fig. 2*B*), the epididymal proteins contained a doublet migrating at a molecular weight of 60–70,000, somewhat smaller than the gp69/71 doublet of the ecotropic RLV (14) and the slightly higher molecular weight gp70 band of the xenotropic NZB virus (15, 16).

Retrovirus Association with Sperm Cells. Sperm in the epididymal lumen of NZB, Swiss, and C57BL/6 males were examined by electron microscopy for the presence of asso-

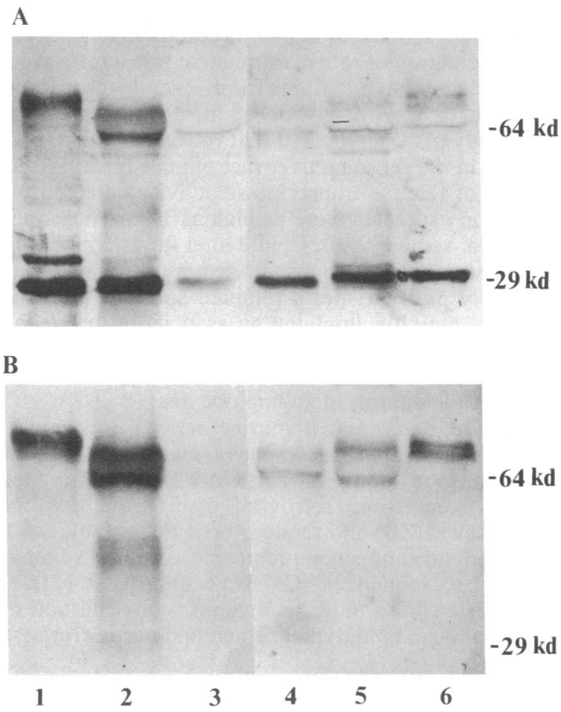

FIG. 2. Immunoblot analyses of seroreactive retrovirus proteins in epididymal fluids of male mice. The filters were blocked, incubated with primary and biotinylated secondary antibody, and developed as described in Fig. 1. Lanes: 1, NZB virus (5×10^6 particles); 2–5, epididymal proteins from NZB (3.5 μg), C57BL/6 (12.8 μg), Swiss (7.0 μg), and AKR (10 μg), respectively; 6, RLV (5×10^6 virus particles). (*A*) Anti-p30. (*B*) Anti-gp70. The dashes indicate the positions of molecular weight markers in kDa (kd): bovine serum albumin (64 kDa) and carbonic anhydrase (29 kDa).

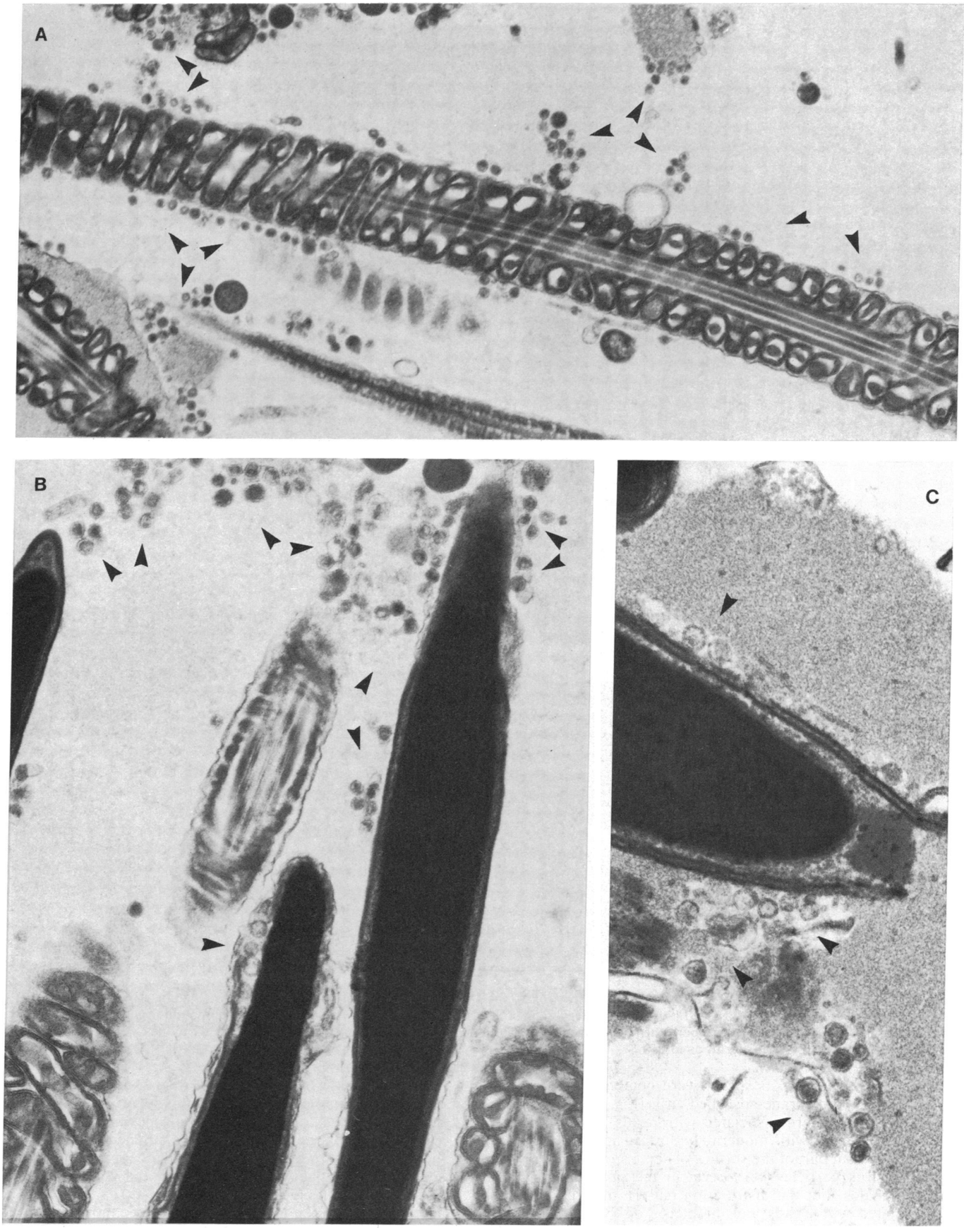

FIG. 3. Sperm-associated retroviruses in the epididymis of NZB and Swiss males. (*A*) NZB sperm midpiece and tail (×21,000). (*B*) NZB sperm heads (×33,000). (*C*) Swiss sperm head (×53,000). Small arrows indicate retrovirus particles.

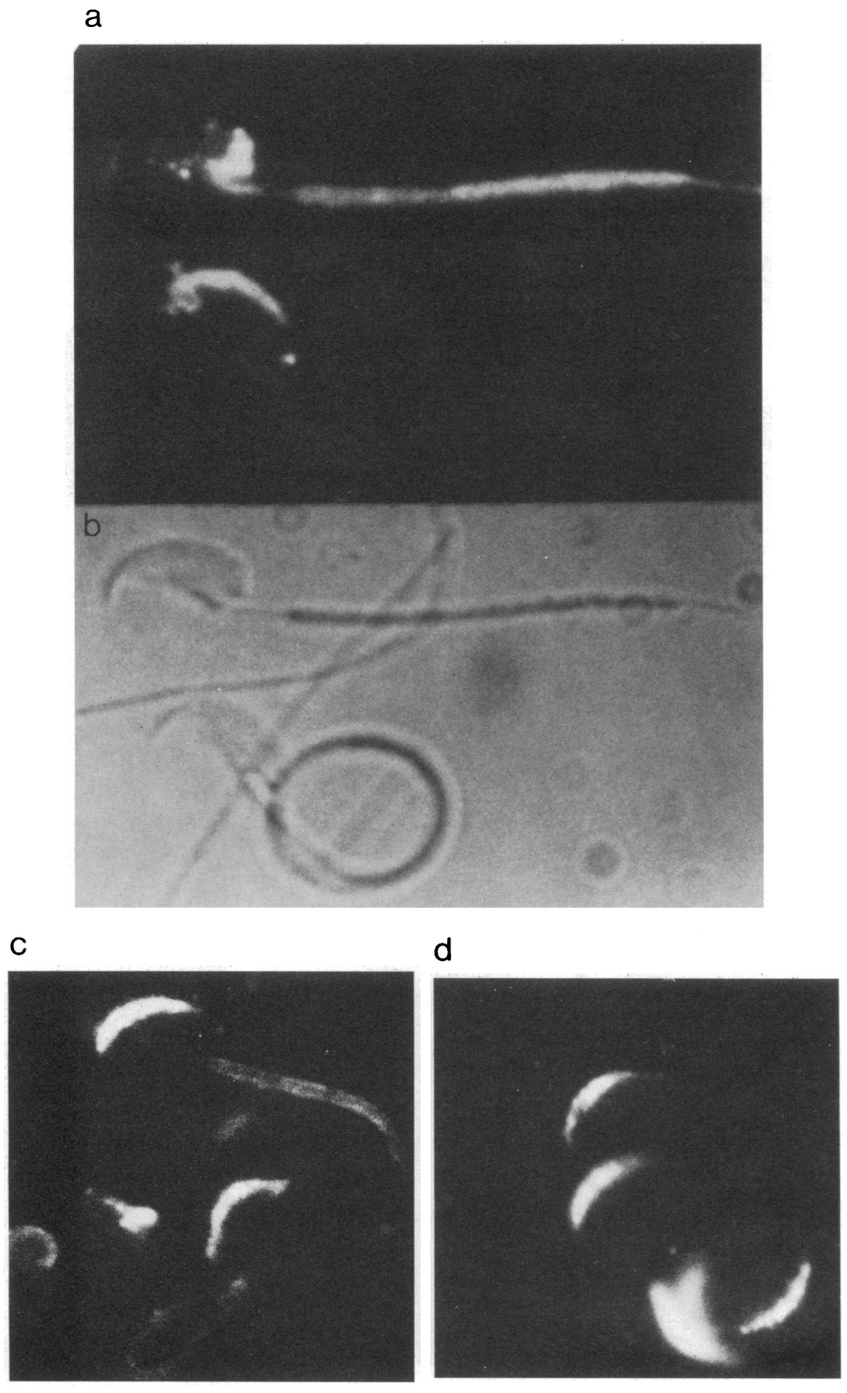

FIG. 4. Immunodetection of sperm-associated retroviruses. (*a*) Fluorescence micrograph of NZB sperm incubated with anti-gp70. (*b*) Bright-field micrograph of sperm in *a*. Fluorescence micrograph of Swiss sperm incubated with anti-p30 (*c*) or nonimmune goat serum as primary antibody (*d*) (the crescent-shaped sperm head fluorescence was due to binding by secondary donkey antibody alone and was absorbed by preincubation with washed sperm, as in *a*).

ciated virus. The NZB sperm were coated with what appeared to be membrane-adsorbed virus (Fig. 3 *A* and *B*). The virus particles were associated with heads, midpieces, and tails, as well as with moderately stained material that appeared to coat part of the surface of some sperm. There were fewer virus particles per sperm in the preparations from Swiss males, but the distribution pattern of virus particles associated with heads and midpieces of Swiss sperm was similar to the NZB (Fig. 3*C*). Virus was only occasionally observed in the epididymal lumen of the C57BL/6 males (data not shown), in keeping with the lower concentration of virus in this strain.

To determine whether or not the virus–sperm associations observed in the electron micrographs were possibly due to fixation artifacts, sperm cells were purified through an albumin cushion, and subsequently examined for associated virus proteins by indirect immunofluorescence (Fig. 4). NZB sperm were routinely positive head to tail when incubated with antibody directed against gp70 (Fig. 4*a*) and p30, in good agreement with the distribution of virus in the electron micrographs. Under the same conditions, the virus proteins were detectable on smaller regions of individual Swiss and AKR sperm cells, principally midpieces, and the indirect immunofluorescence was stronger with anti-p30 antibody

than with anti-gp70 antibody (Fig. 4*c*). The C57BL/6 sperm did not fluoresce under these conditions, probably due to the lower level of virus in this strain.

DISCUSSION

Our experiments demonstrate the presence of retrovirus particles in the epididymides of four strains of mice at sufficient concentration to indicate that retroviruses are commonly expressed in the male reproductive tract of mice and may be an important means of murine retrovirus spread.

That the immunoblot analyses of the epididymal virus proteins were more similar to each other than to the viruses was somewhat surprising. If the epididymal viruses were xenotropic, their seroreactive protein bands would be expected to most closely resemble the NZB virus. Alternatively, if the epididymal viruses were ecotropic, their immunoblot banding patterns might be more similar to RLV, although this possibility may be less likely due to the difficulty in identifying complete ecotropic retroviral gene sequences in the DNA of Swiss or NZB mice (17, 18). The results of the immunoblot analyses suggest that the epididymal viruses may represent specific expression of endogenous retroviral genes in the reproductive tract. Earlier reports of seroreactive but structurally distinct gp70s isolated from serum and seminal fluid of strain 129 mice support this possibility (12). DNA probes specific for endogenous Moloney mink cell focus-inducing virus (MCF) retroviral gene sequences (20) identified more MCF than xenotropic proviral DNA segments in Swiss, NZB, AKR, and 129 mice. This suggests another class of endogenous murine retroviruses that could be expressed specifically in reproductive tract cells.

Epididymal virus would be ejaculated with the sperm and could interact with whatever cells come into contact with the ejaculum. Virus adsorbed to the surface of the sperm would be transported to more distant cells by the motion of the sperm. In the reproductive tract of the female, the cervix and vagina would be exposed to epididymal fluid viruses, and tissues as distal as oviduct and possibly ovary would be exposed to virus adsorbed to the surface of motile sperm. This mode of retrovirus spread has important implications with respect to the venereal transmission of human immunodeficiency virus, particularly if reproductive tract epithelial cells become productively infected with virus.

Adsorption of virus to sperm has been reported for infectious hematopoietic necrosis (IHN) virus and fish sperm (19). The IHN virus–sperm association is thought to be responsible for transmission of IHN virus to offspring. To our knowledge, this is the first report of retrovirus adsorption onto mammalian sperm. The nature of the association, whether specific virus receptor or less-specific membrane adsorption, is not known. Studies to determine the infectivity of the sperm-associated retrovirus, the incidence of transmission to females at breeding, and the effects on fertilization and embryo development are necessary to understand the significance of the male reproductive tract retroviruses.

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