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Direct Exposure of Mouse Spermatogenic Cells to High Doses of Adenovirus Gene Therapy Vector Does Not Result in Germ Cell Transduction

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ABSTRACT

The potential for adenovirus gene therapy vectors to gain access to male germ cells was rigorously tested in the mouse by injecting high titers of the vector directly into the testis and epididymis, or by exposing sperm to the vector immediately prior to or during *in vitro* fertilization. The adenovirus vector carried the bacterial *lacZ* gene (Adβ-Gal) driven by the Rous sarcoma virus (RSV) promoter, and infection was assessed by testing for *lacZ* expression, either with antibodies to LacZ protein or by staining for LacZ enzymatic activity. A total of 10^9 plaque-forming units (PFU) was inserted into the testis or epididymis, and *in vitro* fertilization was performed after sperm were exposed either to 10 or 100 PFU per sperm cell. *lacZ* expression was examined within testes for several weeks after injection, and in preimplantation embryos produced by *in vitro* fertilization with sperm exposed to the gene therapy vector. Direct injection of Adβ-Gal into either the testis or epididymis resulted in *lacZ* expression only within the interstitium of the testis and not within seminiferous tubules. Despite direct exposure of spermatogenic cells or mature sperm to high titers of virus, *lacZ* expression was likewise not detected in embryos. These findings are consistent with the conclusion that the risk is minimal for germ line integration of adenovirus vectors exposed to male reproductive cells.

OVERVIEW SUMMARY

High quantities of an adenovirus vector were injected directly into mouse testes or were placed in contact with mature sperm prior to *in vitro* fertilization. The gene therapy vector encoded the bacterial *lacZ* gene, and infection was assessed by testing for *lacZ* expression in spermatogenic cells or preimplantation embryos derived from *in vitro* fertilization experiments using the sperm exposed to the vector. No expression of *lacZ* was seen in the testis, and more than 600 preimplantation embryos likewise showed no expression, even when sperm were exposed to 100 infectious particles per cell prior to and during *in vitro* fertilization. These experiments provide strong evidence that adenovirus vectors cannot infect spermatogenic cells, and that the risk is low for adenovirus integration into the male germ line during the course of somatic gene therapy.

INTRODUCTION

SOMATIC GENE THERAPY offers great promise for treatment of a variety of inherited and acquired diseases. Interest in this novel and poentially powerful therapeutic tool has led to a rapid proliferation of treatment strategies and vectors for gene transfer. As with all therapeutic approaches, gene transfer is associated with potential side effects and toxicities. However, a potential hazard of gene therapy that is not apparently shared by any other medicinal compound is germ line integration of exogenous DNA. While many drugs can alter the genome through mutagenesis, gene therapy can lead to acquisition of new gene function, and these newly acquired traits can be transmitted to all succeeding generations of offspring.

Our current understanding of gametogenesis and early embryogenesis predicts that germ line integration would rarely if ever occur. Of the millions of sperm released in each ejaculate

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only one reaches the egg, and therefore, insertion of genes into spermatozoa would not be detected if it occurred infrequently (Gordon, 1998). Similarly, of the 400,000 oocytes in the ovary, and of the 400 ovulated during the fertile lifetime of a woman, only a few ever contribute to a new conceptus. After fertilization, the cleaving embryo is encased within the zona pellucida, which presents a significant barrier to penetration of macromolecules or infectious agents during the preimplantation period.

Nonetheless, the diversity of gene therapy vectors available, and the variety of routes of vector administration currently being entertained, raise concern that germ line integration might occur. Such theoretical concerns are augmented by findings of vector DNA in gonads of mice after delivery to tissues remote from the gonads, such as the brain and pleural space (Goodman *et al*., 1996; Kucharczuk *et al*., 1996) or after direct intravenous injection of vectors (Ye *et al*., 1998; Rainov *et al*., 1999). Likewise, in studies involving gene delivery to genitourinary tissues, prostate, and bladder, spread to the gonads was also noted (Timme *et al*., 1998; Wood *et al*., 1999; Paielli *et al*., 2000), presumably via the genitourinary duct system and not the bloodstream. However, all these studies utilized only polymerase chain reaction (PCR) assays to demonstrate vector presence; no evidence of cell transduction or expression of viral genes was provided. Some more direct tests for germ line integration have been made in animal models. Fetal sheep exposed to retroviral vectors manifest vector DNA within gonadal tissue after birth, but the few offspring they have sired are not transgenic for the genetic material (Porada *et al*., 1998; Tran *et al*., 2000). Exposure of mouse zona-free eggs to adenovirus β-galactosidase (Adβ-Gal) followed by fertilization was reported to result in expression of β-Gal in all two-cell embryos and integration of the adenovirus genome in 11% of embryos, with expression in F_1 progeny (Tsukui *et al*., 1996). However, no evidence of transduction was noted if zona-intact eggs were used. Adβ-Gal injected intravenously in mice could not be found in the 814 offspring examined (Ye *et al*., 1998). Similarly, intraprostatic injection of a replication-competent adenovirus resulted in expression and possible replication in the testis, and no germ line transmission was detected in 149 offspring (Paielli *et al*., 2000). However, although these findings support the supposition that germ line integration is unlikely, they involve studies wherein relatively small amounts of vector are presented to germ cells.

Given these circumstances, it is essential that a provocative test system be developed that will sensitively detect even rare insertion of gene therapy vectors into reproductive cells. In this article we report use of such a system for adenovirus gene therapy vectors in males. Adenoviruses are widely used in gene therapy protocols because of their relatively high cloning capacity, their ability to infect nondividing cells, and their amenability to production in high titers (Verma and Somia, 1997). As such, an assessment of the safety of these vectors is of paramount importance. To rigorously test for germ line integration, high titers of such a vector were injected directly into the testes or epididymides of mice, or were exposed directly to mature sperm immediately prior to and/or during *in vitro* fertilization (IVF). Infection was assessed by the production of bacterial β-galactosidase, the gene for which (*lacZ*) was included in the vector as a reporter of expression.

MATERIALS AND METHODS

Adenoviral vectors

A replication-defective recombinant adenovirus (Ad) containing the bacterial β-galactosidase gene (β-Gal) under transcriptional control of the Rous sarcoma virus (RSV) long terminal repeat promoter was prepared as previously described (Stratford-Perricaudet *et al*., 1992). Virus titer, expressed as plaque-forming units (PFU), was determined after expansion and double cesium gradient ultracentrifugation purification by plaque assay in 293 cells. For direct injection of the testis, a similar replication-incompetent Ad containing the herpes simplex virus thymidine kinase gene (HSV *tk*) under control of the RSV promoter, as previously described (Chen *et al*., 1994), was used as vector control.

Intravenous and intratesticular injections

C57/BL6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). Twelve-week-old males were anesthetized with sodium pentobarbital (25 mg/kg) to facilitate vector injection. Depending on the experimental conditions mice received injections of 1×10^9 PFU of vector via the tail vein, or direct injections into the testicle or epididymis with the aid of a dissecting microscope. For tail vein injections anesthetized mice were randomized to receive either Adβ-Gal or AdHSV-tk and were injected via the dorsolateral vein. For testicular and epididymal injections a transscrotal incision was made and the testes were delivered through the wound. The right testis was injected with Adβ-Gal and the left with AdHSV-tk. Intratesticular inoculations were performed with a single pass of a 27-gauge needle, injecting vector in a volume of 100 μl. Intraepididymal injections were performed with the aid of a dissecting microscope by passing a 30-gauge needle through the caput epididymis while injecting vector in a volume of 15 μl.

Mice were killed on days 7, 14, 21, and 28 after vector injection. The testes/epididymides were removed and separated prior to placement in 10% buffered formalin. Fixed tissues were paraffin embedded, cut in 5-μm sections, and prepared for hematoxylin and eosin staining or immunohistochemistry for β-Gal expression (rabbit anti-β-galactosidase IgG; Dako, Carpinteria, CA).

In vitro *fertilization*

For these procedures all mice were obtained from Taconic Farms (Germantown, NY). Animals were maintained under conventional conditions in a 14:10 hr light:dark cycle. Immature (4- to 6 week-old) BDF1 females were superovulated by intraperitoneal injection of 5 IU of pregnant mare serum (PMS; Sigma, St. Louis, MO) followed 52 hr later by 5 IU of human chorionic gonadotrophin (HCG; Sigma). Thirteen hours after HCG injection mature BDF1 males were killed and their caudae epididymides and vasa deferentia were excised and placed in prewarmed KSOM (Erbeck *et al*., 1994) medium (Specialty Media, Phillipsburg, NJ). Sperm were expressed into the medium by gentle pressure with forceps and incubated at 37°C to allow capacitation. A total of 13.5 hr after HCG injection females were killed and their ovaries and oviducts were removed to prewarmed KSOM. Oviducts were subsequently opened in

KSOM supplemented with hyaluronidase (2 mg/ml; Sigma), and cumulus masses were placed at 37°C for 2–5 min to disperse cumulus cells. Oocytes with identifiable first polar bodes were then collected and washed several times in KSOM.

Zona-free inseminations. To maximize fertilization rates and production of embryos that could be examined for *lacZ* expression, a series of experiments was performed wherein oocytes were inseminated after removal of the zona pellucida. For these studies, Tyrode's buffer (Sigma) was supplemented with concentrated HCl (1:250, v/v), and the solution was gently applied to the oocytes with a mouth pipette (Talansky *et al.*, 1998). Immediately after zona dissolution the eggs were immersed in fresh KSOM and subsequently transferred to organ culture dishes for insemination. For these experiments, sperm were expressed from the sex ducts into KSOM containing 1×10^8 PFU of the adenovirus gene therapy vector per milliliters. In these collections, sperm counts ranged from 0.3×10^7 to 1.0×10^7 cells/ml, thus giving an exposure ratio of at least 10 infectious particles/cell. After 1 hr of exposure, sperm were pelleted at $300 \times g$ and washed three times with 3 ml of fresh KSOM. The washed sperm where then applied to the oocytes at a concentration of 10^6 sperm/ml. Insemination were performed in 2 ml of KSOM in an organ culture dish. Fertilization was documented in all IVF experiments by cleavage overnight to the two-cell stage and further development to the morula stage.

Zona-intact inseminations. Zona-free inseminations incur the risk that if incompletely washed, residual adenovirus in the sperm preparation could gain direct access to the oocyte surface. Under these circumstances, LacZ protein staining in embryos could be due to infection of zygotes with adenovirus rather than infection of sperm. This risk is exacerbated by the fact that sperm cannot survive centrifugation at high forces, and thus it is difficult to remove all medium from the loose pellets generated after gentle centrifugation. This problem indeed proved significant (see Results). Accordingly, a series of zona-intact inseminations was also performed. For these experiments, all oocytes that were not degenerated, with their zonae intact, were loaded into the semination dish to which was added 1×10^8 PFU of the gene therapy vector. When sperm (1×10^6/ml) were added to these dishes, the sperm were exposed to 100 infectious particles/sperm throughout the insemination period.

LacZ staining of embryos

LacZ staining was performed on all IVF embryos that cleaved to the morula stage. Preliminary studies showed that this Adβ-Gal vector with the RSV promoter is active at this stage and that LacZ production can be readily documented. For all IVF experiments, positive controls for the staining reaction were included and consisted of a 1- to 6-hr exposure of zona-tree, two-celled embryos to the same viral preparations as were used in IVF. These embryos were then cultured individually in 10-μl microdrops of KSOM covered with mineral oil (Squibb, Princeton, NJ) that had been equilibrated with Earle's balanced salt solution (EBSS, 10% [v/v]; Sigma).

After approximately 48 hr of culture, embryos were pooled in 10-μl microdrops of KSOM under mineral oil. Excess medium was drained with a mouth pipette and replaced with 1.25% glutaraldehyde in phosphate-buffered saline (PBS). The draining and replacement procedure was repeated two additional times, after which the process was reversed in order to replace the glutaraldehyde with KSOM. Embryos were then collected and placed in organ culture dishes that contained 1 ml of LacZ staining solution prepared as follows: 5.5 ml of H_2O, 10 μl of 1 *M* $MgCl_2$, 28 μl of 4 *M* NaCl, 333 μl of 1 *M* HEPES (pH 7.3), 750 μl of 0.03 *M* potassium ferrocyanide, 750 μl of 0.03 *M* potassium ferricyanide, and 7 μl of saturated NaOH. To 1 ml of this mixture was added 26 μl of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), 20 mg/ml dissolved in demethyl formamide. This staining solution was prepared fresh for immediately use prior to each test, and the ferrocyanide and ferricyanide solutions were made fresh every 3 days. To minimize evaporation during staining, the side well of the organ culture dish was filled with 3 ml of distilled H_2O. Embryos were stained overnight at 37°C in a standard tissue culture incubator.

RESULTS

Direct injection of vector into male mice

Examination of testis after intravascular dosing of adenovirus failed to reveal evidence of transduction and gene expression of β-galactosidase. In contrast, intratesticular injection of adenovirus noted transgene expression, but expression was restricted to cells outside the seminiferous tubules, mostly to Leydig cells (Fig. 1A). Expression was widespread on day 7 postinjection, but diminished with time. Only occasional positive cells were found by day 21, and essentially no evidence of β-galactosidase was seen by day 28. There was a lack of significant inflammatory response and no evidence of a negative impact on spermatogenesis was apparent. Injection of Adβ-Gal into the epididymis resulted in transgene expression within the columnar lining of the epididymis and within the testis. However, testicular expression was limited to the interstitial compartment in a more localized pattern and was often associated with a lymphocytic infiltrate. As before, expression diminished to negligible levels by day 21 postinjection. While no β-Gal expression was detected in Sertoli cells or spermatogonia, notable loss of active spermatogenesis was detected beginning by day 14 after vector injection in sections of ~50% of testicles examined (Fig. 1B). In some instances damage was severe enough to destroy all but Sertoli cells; paired epididymal tubules were filled with cellular debris and not spermatids.

Zona-free IVF

Five experiments were carried out in which sperm were exposed to 10 infectious adenovirus particles/cell for 1 hr, washed, and used for zona-free insemination. Table 1 shows results of the individual experiments. Of 252 embryos stained after zona-free IVF all embryos were negative in four experiments (220 embryos), and 5 of 32 were positive in experiment 3 (Table 1). The rare staining of these zona-free embryos is shown in Fig. 2A. We interpret these findings as indicating that sperm did not carry adenovirus to the embryo. Rather, in one experiment, sperm were insufficiently washed, and adenovirus in the

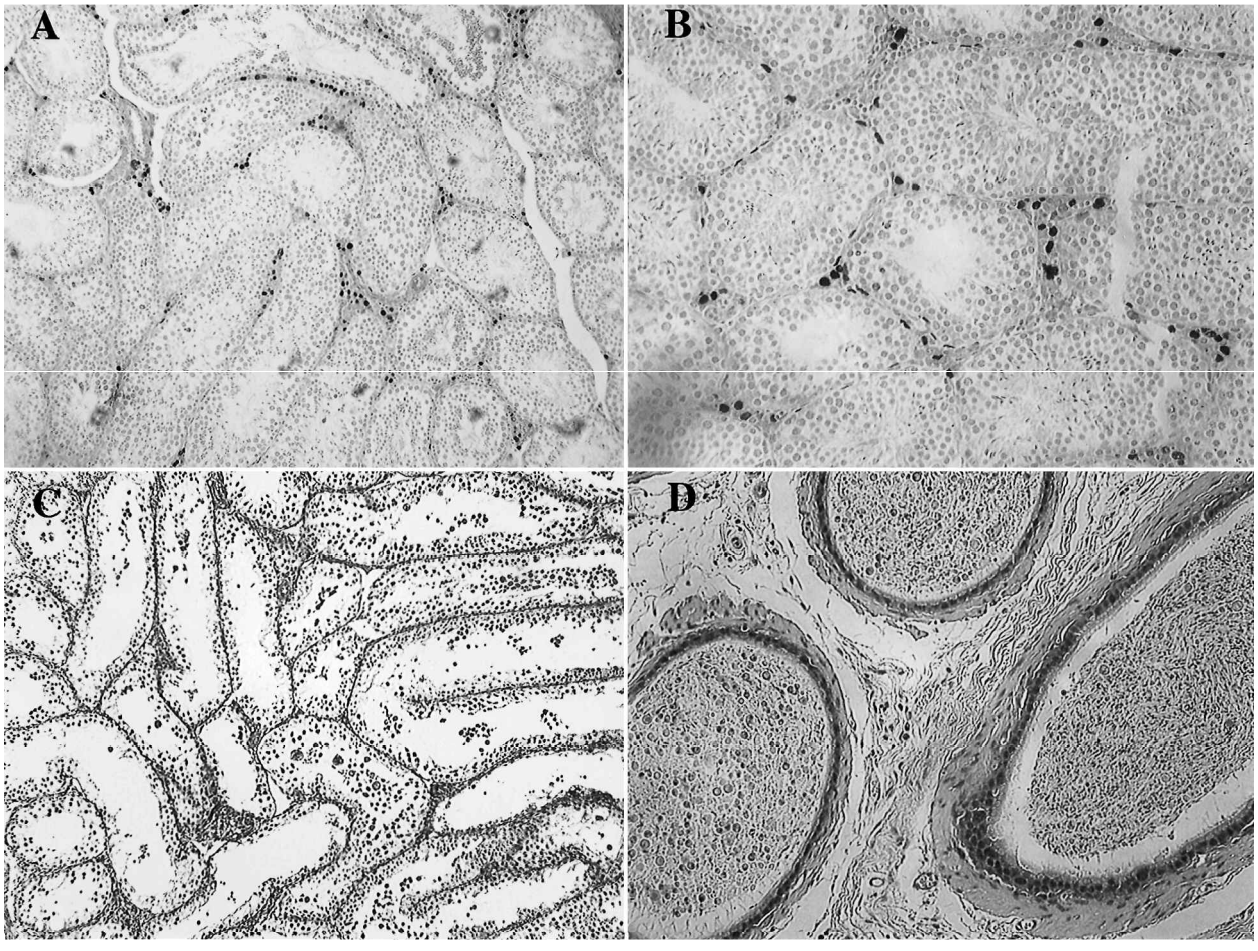

FIG. 1. Photomicrograph of the testis after Adβ-Gal injection. (**A**) Staining for β-galactosidase 7 days after direct vector injection into the testis, illustrating diffuse interstitial expression. (**B**) High-power view of a similar section. (**C**) Hematoxylin and eosin-stained section of a testis 14 days after intraepididymal inoculation of Adβ-Gal, showing significant injury to seminiferous tubules. (**D**) Hematoxylin and eosin-stained section of epididymis 14 days after intraepididymal inoculation of Adβ-Gal. The tubule on the right side contains mature spermatids, while that on the left has only dead cells and debris. Original magnification: (**A** and **C**) $\times$100; (**B** and **D**) $\times$200.

medium, when added to the zona-free insemination, infected the embryos directly. Were adenovirus introduced via the sperm, we would expect some embryos to be stained in every experiment, and we would also predict staining in a higher percentage of embryos.

TABLE 1. IVF USING ZONA-FREE EGGS AFTER EXPOSING SPERM TO ADENOVIRUS AT 10 PFU PER SPERM

Experiment no.	*Positive embryos*
1	0/73
2	0/78
3	5/32
4	0/53
5	0/16
Total:	5/252

Zona-intact IVF

Although we suspected insufficient sperm washing as the cause of rare embryo staining in zona-free inseminations, a low frequency of sperm infection could not formally be ruled out. Accordingly, we conducted 27 experiments wherein zona-intact oocytes were inseminated at a sperm concentration of 1×10^6/ml, with adenovirus present in the insemination at a titer of 1×10^8 PFU/ml, or 100 infectious particles/sperm cell. Virus remained in the insemination dish overnight. Had positive staining in the zona-free inseminations with 10 PFU/sperm been due to sperm infection, the use of 10 times the viral titer in the zona-intact inseminations should have produced a higher frequency of staining.

Figure 2B shows zona-intact embryos produced after insemination in the presence of the adenovirus vector, with five positive control, zona-free embryos. Positive control, zona-free embryos were added at the two-cell stage to the identical viral preparation and incubated for 1 hr. Staining of IVF embryos and the positive controls was performed simultaneously, using

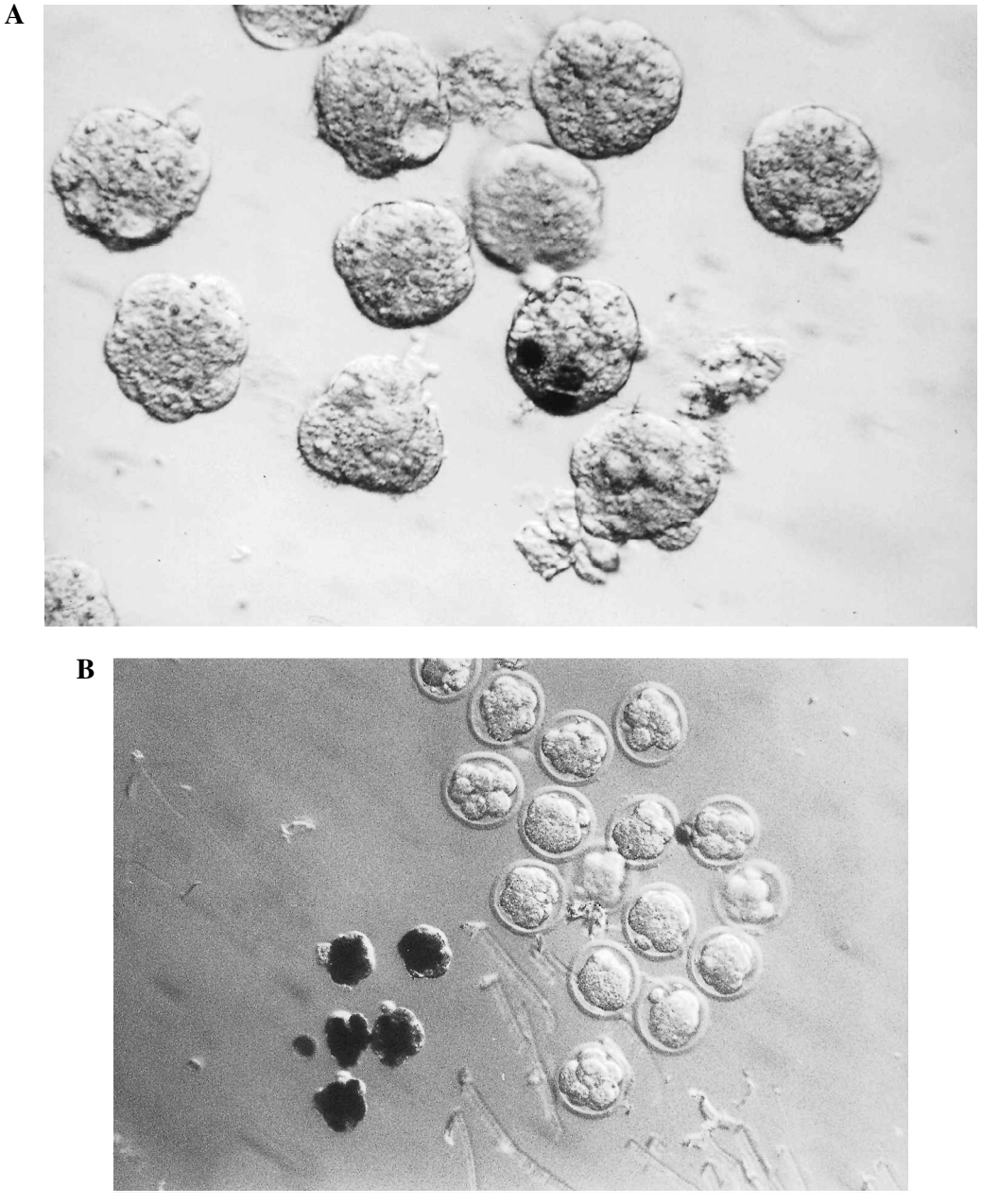

FIG. 2. Embryos produced after IVF, using sperm exposed to Adβ-Gal. (**A**) Morulae produced after zona-free insemination in experiment 3 (see Table 1). Three cells from one embryo show LacZ staining. (**B**) Morulae produced after zona-intact insemination, using 100 PFU of Adβ-Gal per sperm. Five positive controls produced by exposure of zona-free two-cell embryos to Adβ-Gal are at the lower left, with the zona-intact experimental embryos at the right. Note heavy staining of positive controls with complete absence of staining in experimental embryos. Original magnification: (**A**) $\times$200; (**B**) $\times$40.

the same staining solution, and as shown in Fig. 2B the embryos were photographed together. Figure 2B clearly illustrates heavy staining of embryos exposed directly to the vector, with absolutely no staining of the zona-intact IVF embryos.

Of 585 embryos examined after zona-intact insemination, not a single embryo stained positively for LacZ. We therefore conclude that mouse sperm are unable to be infected with adenovirus, and that positive staining in zona-free inseminations was caused by direct infection of embryos due to the absence of the zona barrier.

These data did not rule out the remote possibility that many adenoviruses enter the positive control embryos, but that only a few can be carried to the oocyte via the sperm, and that these smaller numbers of viruses cannot produce enough LacZ for detection. This possibility is addressed in Fig. 2A, which shows an embryo infected with as few as three Adβ-Gal infectious particles, probably due to insufficient washing of zona-free one-celled embryos produced by IVF. As can be appreciated by the staining of only three cells at the morula stage, low levels of infection are readily detectable.

IVF efficiency in the presence of high titers of adenovirus

In the course of performing zona-intact inseminations at 100 PFU/sperm, we observed that the fertilization rate was lower than expected. To explore this finding further, experiments were performed wherein fertilization rates were compared for oocytes inseminated with and without the presence of Adβ-Gal. In these experiments, insemination of 421 oocytes in the presence of adenovirus yielded 74 fertilizations, with a fertilization rate of 18%. In controls inseminated without virus, the fertilization rate was 123 of 265 inseminated, or 46%. This difference is highly significant ($p <<< 0.001$) by χ^2 analysis. We therefore conclude that high quantities of adenovirus impair *in vitro* fertilization in the mouse.

DISCUSSION

Adventitious germ line integration of foreign genetic material in the course of somatic gene therapy is a significant safety concern that must be addressed. We have explored this problem for the male germ line with an adenovirus vector. The male germ line was chosen because direct administration of gene therapy vectors to reproductive organs of males (e.g., prostate) is a common therapeutic strategy, and because sperm are produced continuously rather than once each month. Our approach has been to test provocatively for adenovirus integration into sperm by inserting high quantities of infectious particles directly into the testes of mice, or by exposing sperm directly to adenovirus in the course of IVF. Our findings strongly indicate that adenoviruses cannot infect the sperm, and indicate that gene therapy with this class of vectors poses minimal risk of male germ line integration. We also find that large quantities of adenovirus inhibit fertilization, and accordingly, that one toxic manifestation of adenovirus gene therapy could be at least a transient reduction in fertility.

There are a few gene therapy paradigms that could result in delivery of as high concentrations of virus to spermatogenic cells or sperm as were used in these experiments. Although adenovirus gene therapy vectors can be found by PCR assay in the gonads of experimental animals after intravenous injection (Ye *et al*., 1998), intravenous injection of Adβ-Gal did not result in any detectable intratesticular expression, or in any inflammation that could potentially aid in achieving vector transport into a seminiferous tubule. Since intravenous delivery of adenovirus will result in a high degree of infection within the liver, thereby reducing the amount of vector available to transduce the testis, we chose to inject Adβ-Gal directly into the testicle to ensure the presence of high-dose adenovirus in close proximity to germ cells. However, even when direct injections of the testis were performed, evidence of transduction was limited to the interstitial cells (Fig. 1A). Finally, to mimic the scenario of vector passing in a retrograde fashion from the urinary tract to the testicle, much like that occurring with bacterial epididymoorchitis, Adβ-Gal was injected via the epididymis. Again, evidence of transgene expression was limited to the interstitial cells only. Prior work in rats had noted that injection of Adβ-Gal into rete testes resulted in the transduction of Sertoli cells but not germ cells and in detrimental effects on spermatogenesis (Blanchard and Boekelheide, 1997). We were unable to demonstrate any positive staining within seminiferous tubules, although documentation of interstitial staining after retrograde injection indicates successful placement of vector within the ductule system. Given the size differences between mice and rats, apparent equal volumes of vector used, and demonstration of extraseminiferous tubule staining in the mouse model, the discrepancy may be due to rupture and leakage of vector into the interstitium in the mouse. Escape of vector from the seminiferous tubules by this route could have reduced exposure of Sertoli cells, thus accounting for negative staining of these cells. The important facet of both studies, however, is the complete lack of transduction of spermatogenic cells.

There exist several hypothetical mechanisms whereby some testicular spermatogenic cells may express *lacZ* but that expression escapes detection. Since we did not systematically examine all seminiferous tubules, expression within a few cells might have been overlooked. We consider this possibility remote because we did examine tubules immediately adjacent to interstitial cells that exhibited LacZ staining (Fig. 1). The possibility that insufficient quantities of the virus were administered is again rendered unlikely by the fact that expression within interstitial tissue was readily detected. Another prospect deserving consideration is that function of the Rous sarcoma virus promoter is restricted to cells at a specific stage of gametogenesis. A study of the cytomegalovirus (CMV) promoter in transgenic mice showed expression within late spermatids but not at earlier stages of differentiation (Baskar *et al*., 1996). To our knowledge the activity of the RSV promoter in differentiating sperm has never been thoroughly examined. To avoid missing transient expression at a specific spermatogenic stage, we studied expression over a 4-week time period. The absence of expression throughout the study interval strongly indicates that sperm cell transduction did not take place. However, it is important that further studies with other promoters be performed to confirm these findings.

We were also able to demonstrate significant detrimental effects on spermatogenesis after epididymal injection (Fig. 1B). The inflammatory infiltrate associated with β-galactosidase expression was limited to the extraseminiferous compartment and was localized, making it an unlikely cause of germ cell loss. Moreover, the lack of β-galactosidase expression within Sertoli cells would imply that indirect toxicity to the germinal epithelium secondary to Sertoli cell injury is not a mechanism behind the observed findings. Certainly, direct exposure of sperm to Adβ-Gal in our IVF experiments did not result in a degree of toxicity consistent with the destruction of the germinal epithelium seen after epididymal injection. The finding of interstitial staining, perhaps from tubule rupture and escape of Adβ-Gal into the interstitial space, rather suggests that pressure effects may account for the severe damage to the germinal epithelium. However, since exposure of sperm to high doses of Adβ-Gal did impair IVF, direct toxicity to spermatogenic cells is possible and merits further exploration.

To test further for even rare transduction of sperm by adenovirus, we exposed sperm directly to high titers of Adβ-Gal. This approach constitutes a far more rigorous examination of transduction potential then progeny testing after vector administration to the gonads (e.g., Ye *et al*., 1998; Paielli *et al*., 2000). In progeny testing experiments it is not possible to know the

percentage of sperm giving rise to conceptuses that is actually exposed to the vector, while in our IVF experiments we could be certain that 100% of sperm participating in fertilization were exposed to high quantities of Adβ-Gal. When either 10 or 100 PFU/sperm cell was used for IVF, we could not document introduction of the vector into embryos via the fertilizing sperm.

Although our negative IVF results are compelling, they do not formally rule out the possibility that interspecies differences between mouse and human could allow infection of human but not mouse sperm. Indeed, there has been a report that adenoviruses administered to porcine sperm express *lacZ* in embryos after IVF, integrate, and express their genes in a variety of tissues in piglets born after such embryos are transferred (Farre *et al*., 1999). There are several potential problems with this study, however. The staining procedure employed for the embryos involved exposure to the staining solution for 15 days. Such long incubations predispose to nonspecific deposition of the iron-based stain. Indeed, these investigators reported staining of the zona pellucida (Farre *et al*., 1999), which is outside of the embryo and is inert. Since the embryos were fixed prior to staining, it is not likely that LacZ enzyme would leak from the embryo and become situated at the zona pellucida. In our hands, mouse embryos that express *lacZ* exhibit maximal staining after only a few hours of exposure, with no staining outside the blastomeres.

Another difficulty with this report is that the vector was constructed such that the *lacZ* gene was equipped with a nuclear localization signal (NLS; Bonnerot *et al*., 1987; Herz and Gerard, 1993). This was also the case with the vector used for our study. We find that staining with such vectors is manifest as nuclear staining only (see Fig. 2A). However, nuclei of pig embryos cannot be visualized by light microscopy due to the high quantities of lipid in the embryo. To visualize pronuclei for microinjection, for example, it is necessary to centrifuge the embryo in order to shift lipid to the margin of the cell (Hammer *et al*., 1985). In this report of adenovirus infection of porcine sperm, the embryos were not centrifuged and subcellular compartments such as the nucleus were not visualized either in control or experimental embryos (Farre *et al*., 1999). Still another problem with these data was that negative control and presumptively transduced embryos were not photographed together. Since the difference in staining between control and experimental embryos is slight, it could be accounted for by differences in exposure conditions when photographs were taken.

Yet another unexplained finding in this study was the discrepancy between expression data and Southern blot results. The authors reported expression of the adenovirus vector in heart, kidney, liver, lung, and muscle of piglets born after sperm infection, IVF, and embryo transfer (Farre *et al*., 1999). Since the vector used was replication defective, this result could be explained only by early integration. Integration prior to separation of the cell lineages that give rise to these many tissues should be readily detected by Southern hybridization, yet the study reports negative results after Southern analysis. Given these problems, we conclude that further work is required to confirm infection of pig sperm with adenovirus.

Another approach to evaluating susceptibility of cells to adenovirus infection is to test for the virus receptor. Attachment of adenovirus to the cell surface is mediated by the coxsackievirus and Ad receptor (CAR; Bergelson *et al*., 1997; Tomko *et al*., 1997), while internalization of the virus requires either the $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin molecule (Wickham *et al*., 1993; Bai *et al*., 1994; Nemerow and Stewart, 1999). We know of no study that demonstrates the presence of these integrins on sperm of any species, although other integrin subunits have been detected (Shinohara *et al*., 1999). We are similarly unaware of studies that test for the presence of CAR on sperm, eggs, or embryos. Although such studies are not as direct a test for infection as are tests for expression of vector reporter genes, and although low levels of receptor could escape detection, examination of gametes and embryos for viral receptors could provide useful correlative information.

Of interest was our observation that fertilization is inhibited in the presence of adenovirus. The mechanism underlying this inhibition is unclear. Since infection of the sperm is evidently not the cause, weak binding of virus to the sperm surface or zona pellucida may account for the observation. The possibility that physical interference with sperm–egg interaction is the cause of reduced fertilization is consistent with the finding that fertilization was not abolished. Were cytotoxic effects of virus the cause of fertilization impairment such high titers of virus might be expected to completely block fertilization. Regardless of its underlying mechanism, fertilization impairment by adenovirus could have clinical implications if low levels of virus were capable of interfering with sperm penetration. We have not yet conducted experiments to determine the amount of virus required to affect fertilization in the mouse. However, such studies are clearly required for a better characterization of this potentially toxic effect.

In summary, aggressive attempts to introduce adenovirus vectors into sperm in mice have failed to yield any evidence of infection. These findings should provide new confidence that somatic gene therapy with adenovirus vectors poses a negligible risk of male germ line integration.

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