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Technical Article

Transplantation of testis germinal cells into mouse seminiferous tubules

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ABSTRACT In the adult male, germ cell differentiation takes place in the seminiferous tubules of the testis by a complex, highly organized and very efficient process. A population of diploid stem-cell spermatogonia that lie on the basement membrane of the tubule continuously undergoes self-renewal and produces progeny cells, which initiate the process of cellular differentiation to generate mature spermatozoa. Each testis contains many seminiferous tubules, which are connected at both ends to a collecting system called the rete testis. The mature spermatozoa pass from the tubules into the rete and are then carried through efferent ducts to the epididymis for final maturation before they are ready to fertilize an egg. In previous studies, we have demonstrated that donor testis cells collected from a fertile mouse are able to generate spermatogenesis when transplanted to the seminiferous tubules of an infertile male. The spermatozoa produced by the recipient from the donor-derived spermatogonial stem cells are able to fertilize eggs and produce progeny carrying the donor male haplotype. Furthermore, donor testis stem cells from a rat will generate normal rat spermatozoa following transplantation to a mouse testis. The spermatogonial transplantation technique is clearly valuable and applicable to many species, but it is difficult. Therefore, several procedures to introduce donor cells into the seminiferous tubules of a recipient have been developed using the mouse as a model, and they are described here in detail. The results indicate that microinjection of cell suspensions into the seminiferous tubules, efferent ducts or rete testis are equally effective in generating donor cell-derived spermatogenesis in recipients. Each approach is likely to be useful for different experimental purposes in a variety of species.

KEY WORDS: *testis, spermatogonia, stem cell, transplantation, mouse*

Introduction

Spermatogenesis is central to the continuation of any species. Consequently, it is not surprising that it should be highly efficient and resistant to damage. Daily sperm production from males of different mammalian species varies over a wide range from 0.13×10^9 for the human to 16×10^9 for the boar (Sharpe, 1994; Barratt, 1995). Considering that only a single or a few female germ cells must be fertilized during each reproductive cycle, male gamete production is very high. Furthermore, whereas eggs, the female germ cells, are produced intermittently for union with their male counterpart, spermatogenesis is continuous throughout the adult life of males in most mammalian species. The high level of male gamete production results from the unique anatomy, rigid organization and temporal regulation of the process (Bellvé, 1979; Ewing *et al.*, 1980; Russell *et al.*, 1990).

Spermatogenesis takes place in the seminiferous tubules, which measure approximately 200 microns in diameter in the mouse, and the estimated 15 to 20 tubules of the mouse testis have a combined length of about 2 meters (Bascom and Osterud, 1925; Setchell *et al.*, 1994). In the human, there are 250 to 1,000 tubules in a testis, each tubule with a length up to 80 cm (Bascom and Osterud, 1925; Dym, 1983), for a total of about 250 meters of seminiferous tubule per testis. In the rat and mouse, each tubule enters the rete testis, a collecting area, located on the surface of the dorsal aspect of the testis just beneath the tunica albuginea (Roosen-Runge, 1961; Dym, 1976). This structure underlies the vascular pedicle carrying the arteries, veins and lymph ducts to the testis and measures approximately 1.5 mm in length, 0.5 mm in width and 0.1 to 0.2 mm in depth (Dym, 1976; our personal observations). From the rete emerge 3 to 6 efferent or collecting ducts, which course toward and enter the head of the epididymis. The ducts carry spermatozoa formed in the seminiferous tubules to the epididymis where final maturation occurs. Transport of mature spermatozoa to the epidi-

Abbreviations used in this paper: DMSO, dimethyl sulfoxide; *LacZ*, *E. coli LacZ* gene; EDTA, ethylenediaminetetraacetic acid; HBSS, Hanks' balanced salt solution; X-gal, 5-bromo-4-chloro-3-indolyl ß-D-galactoside; ZF, zinc finger.

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dymis, following release into the seminiferous tubule, is believed to be quite rapid (Russell, 1984).

Within the seminiferous tubules the germ cells are associated in a highly organized and rigidly determined architecture (Huckins, 1971; Clermont, 1972; Russell *et al.*, 1990; de Kretser and Kerr, 1994). There are three main phases of spermatogenesis. In the first, known as the mitotic phase, spermatogonia proliferate to increase dramatically in number and ultimately produce primary spermatocytes (de Rooij *et al.*, 1989; de Kretser and Kerr, 1994). The largest increase in cell number occurs during this phase. There are three types of spermatogonia: stem cell spermatogonia, proliferative spermatogonia and differentiating spermatogonia (Russell *et al.*, 1990). The first two types are believed to undergo self-renewal. The proliferative spermatogonia simultaneously produce a population of differentiating spermatogonia which are irreversibly committed to proceed through the complicated process of spermatogenesis and generate mature spermatozoa, which takes 35 days in the mouse. It is the self-renewal of the early spermatogonial cells that is the foundation of spermatogenesis and allows it to continue throughout most of adult life. These self-renewing cells are generally referred to as stem cell spermatogonia. The second phase of spermatogenesis is meiosis, in which primary spermatocytes undergo recombination of genetic information and generate round spermatids (Stern, 1993). In the third phase of spermatogenesis, known as spermiogenesis, round spermatids undergo a complicated differentiation process that results in the characteristic species-specific morphology of mature spermatozoa (Clermont *et al.*, 1993; Meistrich, 1993). No cell division occurs during this phase. Overall, the process is very productive; theoretically a single stem cell in the rat is capable of producing 4096 mature spermatozoa (Russell *et al.*, 1990). However, degeneration of cells occurs at every stage of differentiation, and overall efficiency has been estimated at 10 to 25 percent (Tegelenbosch and de Rooij, 1993; Barratt, 1995). Nonetheless, large numbers of spermatozoa are produced by all species, and enormous numbers by some males (e.g. the boar). While the efficiency of the process is affected by internal and external phenomena, the cellular associations and temporal regulation are rigidly maintained and characteristic for any species (Ewing *et al.*, 1980; Russell *et al.*, 1990; de Kretser and Kerr, 1994).

Germ cells are supported by somatic Sertoli cells, which are evenly spaced in the seminiferous tubule and attached to the basement membrane (Russell, 1993). Between adjacent Sertoli cells there exist tight junctions that separate the adluminal compartment of the tubules, which contains the advanced stages of germ cells with unique antigens, from the basal compartment occupied by spermatogonial stages (Dym, 1983; Russell, 1993). This separation of compartments is often referred to as the "blood-testis" barrier (Dym, 1983; Byers *et al.*, 1993). Passage of germ cells through the Sertoli cell junctions occurs at the primary spermatocyte stage in a complex cellular process which maintains the separation of the basal and adluminal compartments. Germ cell nutrition and regulation of spermatogenesis are thought to be significantly influenced by Sertoli cell activity (Enders, 1993; Bardin *et al.*, 1994). Thus, the Sertoli cell responds to many factors, including the trophic hormones, follicle stimulating hormone and luteinizing hormone, as well as to steroid hormones, particularly androgens, to modify the process of spermatogenesis (Griswold, 1993; Sar *et al.*, 1993; Sharpe, 1994). Furthermore, the germ cell stages in a particular section of seminiferous tubule affect the appearance and function of the Sertoli cells (Morales and Clermont, 1993). The result is a complicated interactive process between hormonal milieu and cellular influences that produces in a highly organized pattern of cellular associations that is closely regulated, highly efficient, and characteristic for each species. Spermatogenesis is a complex and an essential process, about which there remain many unanswered questions.

One approach to understanding spermatogenesis is to manipulate the initiation and development of the process. Our recent reports of successful transplantation of mouse donor testis cells from a fertile male to the seminiferous tubules of an infertile recipient male, in which the donor cells generate spermatogenesis, provides an ideal technique to address many questions about the process in a unique manner (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994). In addition, subsequent experiments have demonstrated that transplanted rat testis cells will generate spermatogenesis in the testes of immunodeficient mice, suggesting that the cells of many species may be successfully transplanted (Clouthier *et al.*, 1996). Xenogeneic transplantation has the potential to greatly extend the range of questions addressed, since it permits the study of interactions between germ cells and Sertoli cells from different species. This type of study would be particularly valuable as a means to identify whether germ cells or Sertoli cells control the duration of spermatogenesis and the distinct cellular associations characteristic of different species. The ability to cryopreserve spermatogonial stem cells will facilitate both transplantation and culture studies (Avarbock *et al.*, 1996).

There are several obstacles to efficient utilization of spermatogonial transplantation to address important questions regarding spermatogenesis. First is the technical difficulty of the procedure. Based on the anatomy of the testis and collecting duct system, several possible approaches exist to introduce donor cells into the lumen of the seminiferous tubules of a recipient mouse testis. The first and most obvious is to inject a cell solution directly into the seminiferous tubule and fill each tubule individually. Often, flow of the solution from an injected tubule through the rete testis will fill partially or completely a variable number of other tubules. Thus, a few sites of injection may fill a large number of tubules. A second approach is to inject directly into the rete testis, to which all tubules are connected at each end, thus in theory filling all tubules from one injection site. A third possibility is to cannulate one of the fine efferent ducts running from the rete testis to the head of the epididymis, thereby filling the rete and subsequently the tubules. Each of these approaches appears feasible and applicable to many species. Therefore, we have examined the techniques necessary to achieve each successfully in the mouse, and report here the results of our studies. The findings will be useful to those undertaking spermatogonial stem cell transplantation in the mouse and can be extended to testis cell transplantation in other species.

Experimental Protocols

Choice of animals

Several combinations of donor and recipient mice can be used for spermatogonial transplantation experiments. It is important that the recipient be immunologically tolerant to the donor testis cells.

While the adluminal compartment of the seminiferous tubules is protected from both cellular and antibody immunological responses of the host, the basal compartment, where the donor stem cells must establish themselves, is not protected from host rejection mechanisms by the Sertoli cell "blood testis" barrier. However, additional immunological protective mechanisms are associated with Sertoli cells (Tung, 1993; Bellgrau *et al.*, 1995). It has been demonstrated that Sertoli cells are able to inhibit host immunological rejection of foreign cells. One mechanism by which the Sertoli cell achieves this is the presentation of CD95 ligand, a surface antigen as well as a soluble molecule, which causes destruction of activated T lymphocytes expressing high levels of CD95 (Bellgrau *et al.*, 1995). Nonetheless, the effectiveness of these mechanisms regarding transplanted testis cells is not clearly defined; therefore, immunological compatibility of donor and host removes these factors from consideration in assessing experimental results.

In previous experiments, the transplantation of C57BL/6 donor testis cells to recipient homozygous W mice has been shown to be effective (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994). These recipient mice do not have endogenous spermatogenesis, because they lack the c-kit receptor essential for male germ cell proliferation. The absence of endogenous germ cells is convenient. However, analysis of recipient mice for donor cell-derived spermatogenesis requires serial section of the testes, which is laborious. In contrast, the use of donor mice that contain a reporter transgene expressed in male germ cells allows detection of transplanted cells by staining techniques and greatly facilitates analysis of recipient testes for stem cell colonization. A transgenic mouse line with a reporter transgene composed of a zinc finger (ZF) promoter fused to the *E. coli LacZ* structural gene *(LacZ)* results in the production of ß-galactosidase in round spermatids and later cell stages of spermatogenesis (Brinster and Avarbock, 1994; Zambrowicz *et al.*, 1994). These mice have a C57BL/6 x SJL genetic background; therefore, their testis cells can be transplanted into C57BL/6 x SJL F1 hybrid mice without concern for immunological interference. The presence of donor cells can be readily determined by incubation of whole testes, or histological sections of testes, in 5-bromo-4-chloro-3-indolyl ß-D-galactoside (X-gal) as previously described (Brinster and Avarbock, 1994; Rhim *et al.*, 1994). While other combinations of donor and recipient could be used, in the experiments described here ZF*LacZ* donor cells were transplanted into C57BL/6 x SJL F1 recipients to compare the success of several transplantation procedures.

Preparation of recipient mice

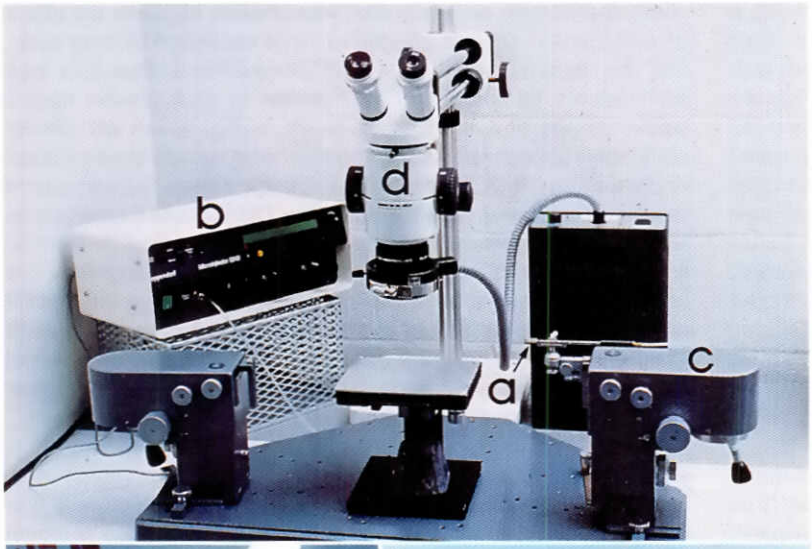
In a normal testis, access to the basal compartment of the seminiferous tubule by donor stem cells introduced into the lumen is limited. The multiple layers of differentiating germ cells as well as the Sertoli cell barrier would interfere with migration of the stem cell from lumen to basal membrane. To provide access to the basal compartment and to reduce competition from endogenous germ cells, several techniques can be used to reduce or destroy endogenous spermatogenesis in recipient males. Effective methods include radiation (Withers *et al.*, 1974; Meistrich *et al.*, 1978; van Beek *et al.*, 1990), chemotherapeutic drugs (Bucci and Meistrich, 1987; Brinster and Avarbock, 1994), experimental cryptorchidism (Nishimune *et al.*, 1978; Mendis-Handagama *et al.*, 1990), cold ischemia treatment (Young *et al.*, 1988), and vitamin A-deficiency (van Pelt *et al.*, 1996). For the experiments described here, the chemotherapeutic drug busulfan was chosen because the effect on endogenous spermatogenesis can be controlled by drug dose, and the treatment can be easily administered. Busulfan was administered by intraperitoneal injection to mice greater than 6 weeks of age at a dose of 38 to 40 mg/kg, which will almost completely abolish spermatogenesis in most mouse strains (Bucci and Meistrich, 1987; Brinster and Avarbock, 1994). The compound was first dissolved in dimethyl sulfoxide (DMSO) and then an equal volume of sterile distilled water added to provide a final concentration of 3.8 to 4.0 mg/ml. The limit of solubility for busulfan is near this concentration, and precipitation is delayed by mixing the DMSO and water phase just before use and maintaining the solution at 35 to 40°C. Following busulfan treatment, the males are maintained for at least one month before testicular cell transplantation to allow endogenous germ cells to disappear from the tubule lumen.

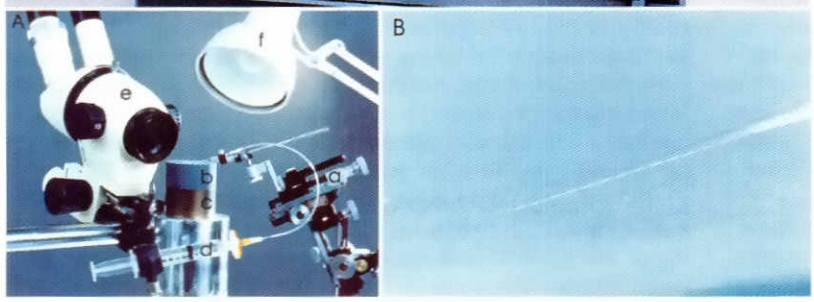
Donor cell preparation

Cells for transplantation are obtained from testes of mice 5 to 60 days after birth by a two-step enzymatic digestion protocol (Bellvé *et al.*, 1977; Bellvé, 1993; Brinster and Avarbock, 1994). In this procedure the tunica albuginea is first removed or peeled from the testis, thereby exposing the seminiferous tubules. The testes are then incubated in approximately 10 volumes of Hanks' balanced salt solution without calcium or magnesium (HBSS) containing 1 mg/ml collagenase (Type IV, Sigma) at 37°C with gentle agitation for 15 min or until the tubules separate. Dispersion of the tubules can be hastened by careful dissection, spreading of the tubules, and removal of intertubular cellular strands when visible. The addition of DNAse (200 to 500 µg/ml) will facilitate intertubular cell dispersion. The testis tubules are then washed 2 to 4 times in 10 volumes of HBSS, followed by incubation at 37°C for 5 min in HBSS containing 1 mM EDTA and 0.25% trypsin. Separation and dispersion of the tubule cells can be hastened by pipetting and gentle agitation. When most of the cells are dispersed, the action of trypsin is terminated by adding a 10 to 20% volume of fetal bovine serum. The inclusion of 200 to 700 µg/ml of DNAse may decrease stickiness and facilitate dispersion of the cells. Following digestion, any large pieces of undigested material are removed, and the cell suspension is filtered through a nylon mesh with 60 µm pore size to remove large clumps of cells. The filtrate is centrifuged at 600xg for 5 min at 16°C and the supernatant carefully removed from the pellet. The cells in the pellet are then resuspended in sperm cell medium (Brinster and Avarbock, 1994) or Dulbecco's modified Eagle's medium containing 10% fetal bovine serum to the volume and/or cell concentration used for injection. The cell volume needed to inject both testes of a mouse ranges from 0.1 to 0.5 ml depending on the injection method. Cell concentrations of up to about $300x10^6$ cells per ml can be used. Concentrations above $150x10^6$ cells/ml are associated with high levels of clumping and plugging of pipettes, which may be reduced by the addition of DNAse at the time of injection. The cells are maintained at 5°C until the time of loading into an injection pipette, usually 1 to 4 h.

Microinjection equipment

Because of the small size of the seminiferous tubule, efferent duct and rete testis, microinjection of donor testis cell suspensions is greatly facilitated by apparatus to visualize the structures and introduce the injection pipette. Figure 1 illustrates a typical experi-





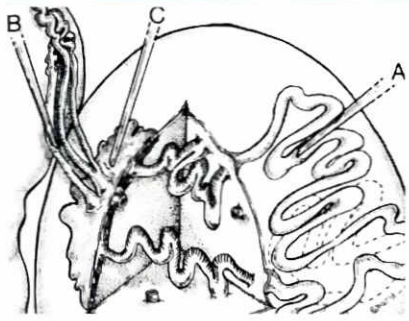


Fig. 1 (Top). Equipment used to inject cell suspensions. *An anesthetized mouse with the testis exposed is placed on the central platform, which should be movable to allow positioning of the mouse and alignment of the microinjection capillary holder* **(a)**. *The injection pipette is secured in the capillary holder which is connected to an Eppendorf pressure injector* **(b)**. *Movement of the pipette is controlled with a Leitz micromanipulator from either the right or left* **(c)**. *The operating field is illuminated with a fiber optic ring light attached to the objective lens of a dissecting microscope* **(d)**.

Fig. 2 (Middle). Micropipette preparation. *A 7.5 cm length of borosilicate glass tubing (0.75 mm internal diameter and 1.0 mm external diameter) is drawn on a Kopf pipette puller (not shown) to form two pipettes 3.5 to 4.0 cm in length with thin pointed tips.* **(Panel A)** *The pipette is placed in an instrument holder of a Narishige micromanipulator (a) with the tip resting on the surface of a grinding stone (b). Rotation speed of the stone is altered by varying the air pressure within the cylinder of an air driven pipette sharpener (c). The shaft of the pipette is connected to polyethylene tubing (PE100, Internal diameter 1 mm) containing filtered water under pressure created by gently depressing the plunger of a 10 ml syringe (d). The tip of the pipette is viewed at 6 to 12 X using a dissecting microscope (e). Illumination of the grinding stone surface is achieved with an incandescent 25 W lamp (f)* **(Panel B)** *A micropipette nearly completely formed. The tip remains clean and open because of the small flow of water. The time required for grinding a micropipette tip is modified by varying the rotation speed of the stone and the pressure of the tip on the stone surface.*

Fig. 3 (Bottom). Microinjection pipette insertions for introducing cell suspensions into the seminiferous tubules. *A seminiferous tubule may be injected directly by inserting the micropipette (~40 μm) into a straight stretch of the tubule (A). The injected solution will flow in both directions from the tip, but the forward movement of the solution is generally greatest. The fluid flows through the tubule, often reaches the rete and fills other tubules. A second approach for introduction of a cell suspension into the seminiferous tubules is to insert the micropipette into an efferent duct between the testis and the head of the epididymis (B). The pipette tip (~40 μm) is gently threaded into a large duct for 3 to 5 mm toward the testis. Pressure in the pipette will cause the cell suspension to flow into the rete and fill most or all of the tubules. A third approach for introduction of a cell suspension into the seminiferous tubules is to insert the tip (~60 μm) of a micropipette into the rete testis (C). This is a small pond-like area beneath the surface of the tunica into which the ends of each seminiferous tubule empty. Thus, the cell suspension potentially has access to all tubules.*

mental arrangement (Brinster and Zimmermann, 1994). The central platform on which the recipient male is positioned should be movable. This allows adjustments to be made in the location of the testis in order to enter the structure to be injected. The injection micropipette is secured in a pipette holder (WPI Instruments catalogue no. MPH6S). The tip of a 1 ml plastic syringe barrel, which has been cut at the 0.4 ml mark, is inserted into the other end of the WPI pipette holder (Brinster and Avarbock, 1994). The cell solution to be injected is deposited in the syringe barrel, which is then screwed onto the metal end of an Eppendorf capillary holder attached by flexible tubing to a pressure injector (Eppendorf model 5242). The Eppendorf capillary holder is secured in a Leitz micromanipulator either on the right or left side of the platform (Fig. 1). Injection rate of the cell suspension is controlled by the pressure injector. The testis is visualized with a dissecting microscope in a magnification range of 6x to 20x and is illuminated by a quartz fiber optic ring light. While the apparatus pictured in Figure 1 is most appropriate for transplantation procedures, it is possible to secure the injection pipette in polyethylene tubing (inside diameter 1 mm), connected to a 1 ml syringe. In this case, the injection rate and flow of cell suspension is regulated by thumb pressure on the plunger of the syringe, and the injection pipette is introduced by hand.

A critical component of the equipment is the injection pipette, which is constructed from a 3 inch length of borosilicate glass with internal diameter 0.75 mm and external diameter 1 mm (World Precision Instruments # TW100-3). The glass tubing is drawn on a Kopf pipette puller (Model 750) creating two potential injection pipettes. The tip of each pipette is ground to a sharp beveled point as shown in Figure 2A and B. A grinding stone is rotated by air pressure using an apparatus previously described (Vurek *et al.*, 1967). The speed of rotation is changed by adjusting the air pressure, and the rate of grinding can be modified by the texture of the stone surface, pressure on the pipette, and speed of stone rotation. The pipette is held in a Narishige micromanipulator and advanced slowly. While grinding proceeds, the fine glass tip is kept clean by maintaining a small flow of filtered distilled water through

tubing connected to a 10 ml syringe (Fig. 2A). By adjusting the rotation speed of the grinding stone and pressure of the pipette on the surface, a suitable pipette can be prepared in 1 to 3 minutes. A tip with an external diameter of 40 to 60 μm is used. Tip angles of approximately 30° are generally best for injection, but a range of angles can be made and stored in a closed box. For storage, pipettes are pressed into two parallel stripes of clay 1 cm apart. The filtered water in the pipette is expelled just prior to injection by the cell suspension entering and filling the pipette.

Seminiferous tubule injection

Perhaps the most obvious approach for introduction of donor cells into the seminiferous tubule is direct microinjection into the lumen. In this procedure, the abdomen of the recipient is opened with a 1.5 cm midline incision, the male placed on the platform (Fig. 1), the right or left testis withdrawn from the body cavity, then positioned on a thin sterile cardboard drape that secures and stabilizes the testis. A small (~3 mm) incision is made in the tunica, and the animal is rotated to align a long, straight stretch of tubule with the injection pipette. The pipette is advanced with the micromanipulator and inserted into the seminiferous tubule (Fig. 3, Pipette A), and pressure in the injection tubing is raised until the cell suspension flows into the lumen. Filling of the tubules can be monitored by observing movement of the cell suspension, which is facilitated by adding a small amount of trypan blue to the injection medium. When flow stops, another tubule is selected and the procedure repeated until all tubules are filled or flow cannot be achieved in the remaining areas. Several incisions in the tunica may be required. Between 50 and 100% of surface tubules can routinely be filled by this procedure. In general, a volume of 0.4 to 0.5 ml of cell suspension is required per animal with this method.

The injection procedure using the seminiferous tubules is shown in Figure 4. A small incision in the tunica results in bulging of the tubules through the opening (Fig. 4A) and some distension of small blood vessels may occur resulting in hemorrhage. Initially, the tubules are easy to move and will slip away from a pipette tip. If the surface dries slightly, penetration is facilitated. A straight section of tubule is entered with the pipette containing the donor cell suspension (Fig. 4B) and pressure in the tubing increased until fluid flows into the tubule. Distant parts of this tubule fill first, and after the injection suspension reaches the rete, other tubules are filled (Fig. 4C). Eventually many or all the surface tubules can be filled with the cell suspension. The time necessary to achieve maximum filling of a testis ranges from 5 to 30 minutes depending on the patency of the tubules, concentration of cells in the injection suspension, and the number of tubules that must be entered. The incisions in the tunica are not sutured, and occasionally adhesions occur between the tubules and epididymal fat, particularly if trauma is great. Following injection the testes are returned to the body cavity, the muscle layer sutured with interrupted silk stitches (size 6-0), and the

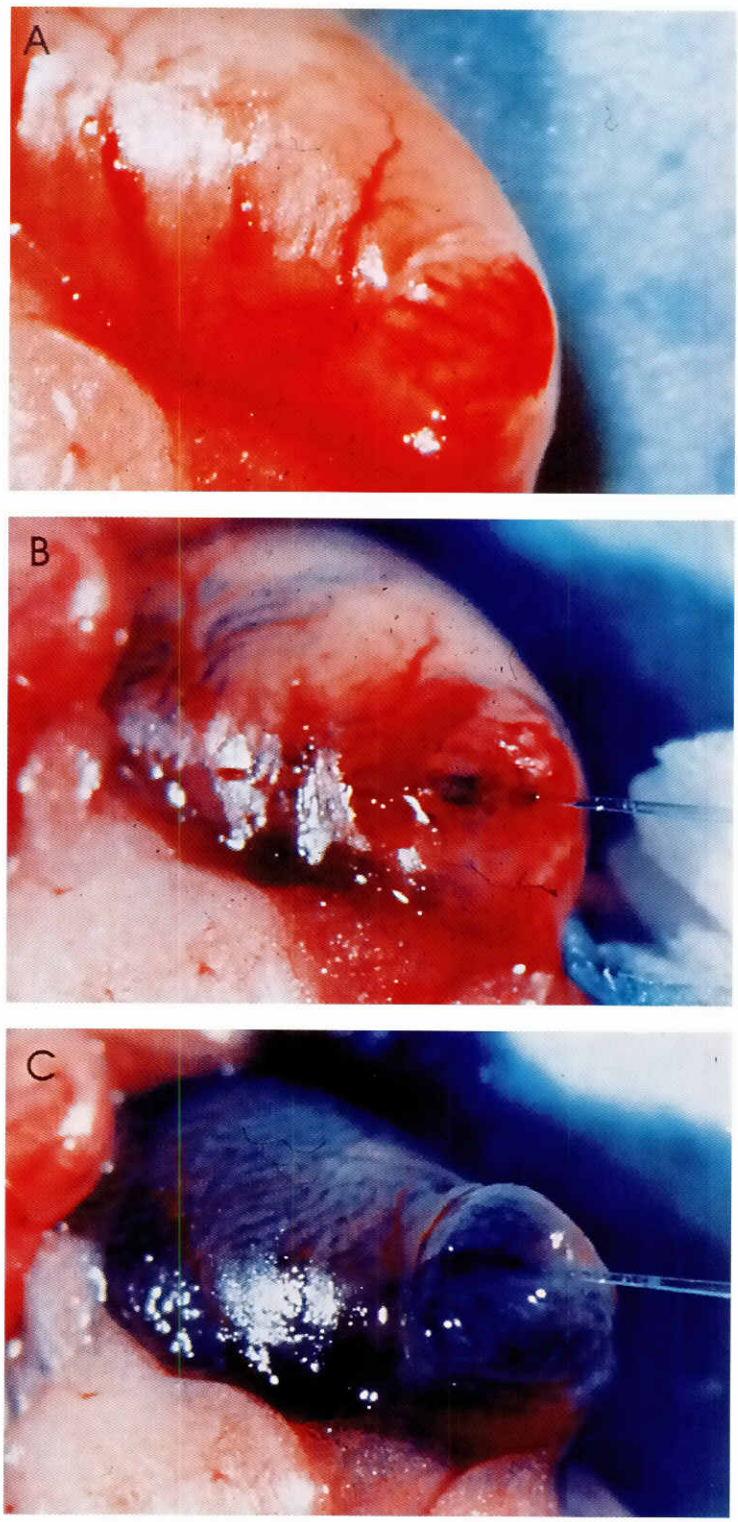
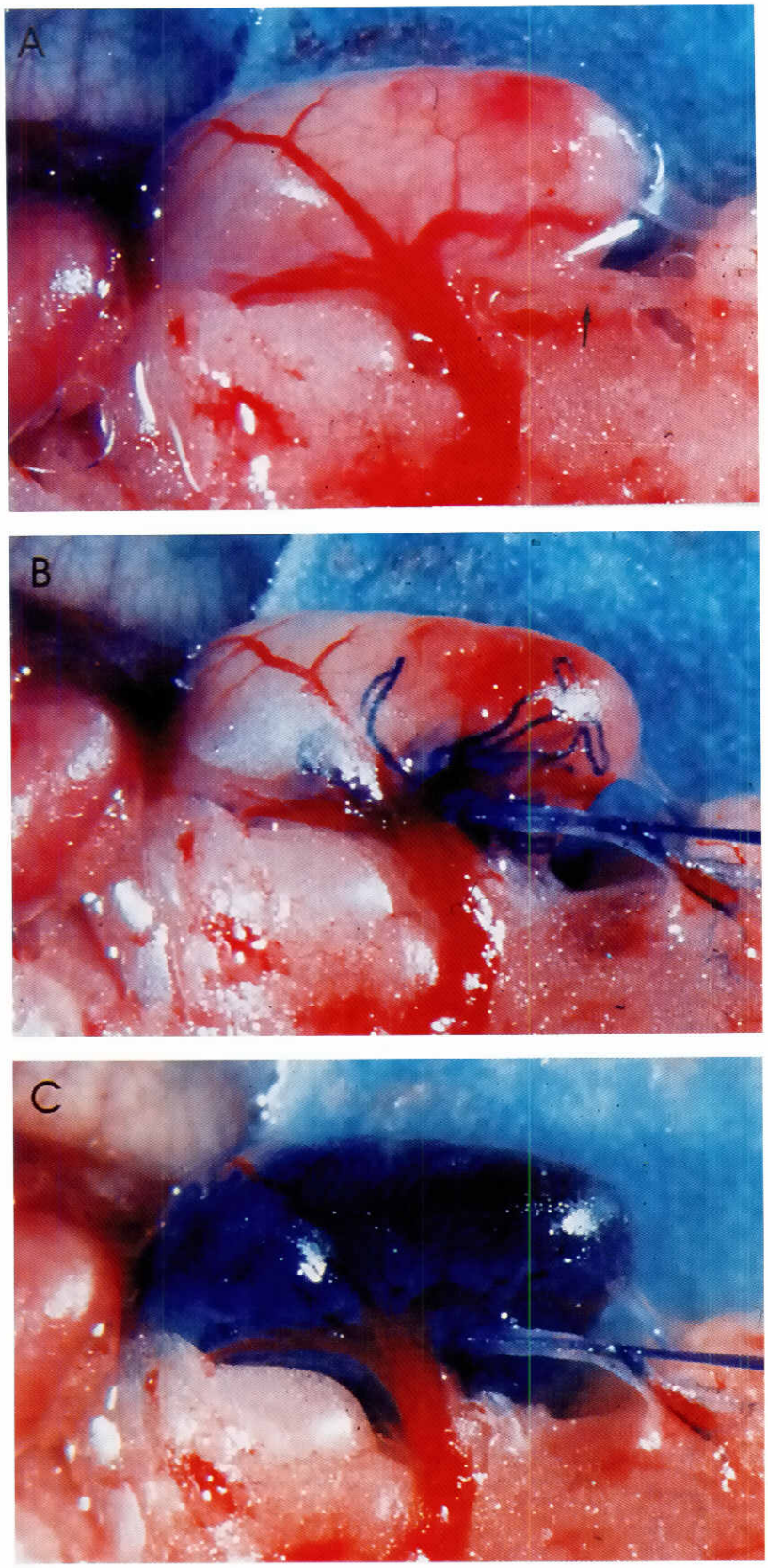


Fig. 4. Seminiferous tubule injection. *In this technique the micropipette is inserted directly into the lumen of the seminiferous tubule.* **(A)** *A small incision has been made in the tunica of a testis from an animal previously treated with busulfan, and several loops of tubule have been extruded. Some local vascular distension and hemmorhage may occur.* **(B)** *A straight section of tubule is selected, and the tubule punctured by the tip of the pipette. A solution containing dye has been injected, and a tubule is shown filling with the solution.* **(C)** *The testis is seen at the conclusion of injection. Almost 100% of the surface tubules are filled with the injected solution. Because each tubule is convoluted and follows a path on the surface and in the interior, surface filling is a good indication of the degree to which the testis tubules have been filled by the injected solution. Dissection time was approximately 10 min, and tubule filling time less than 10 min.*



skin closed with metal wound clips. In our experience surgical approach through the scrotum is less efficient.

Efferent duct injection

Before donor cells can be injected into the seminiferous tubules through the efferent ducts, careful dissection of the area between the testis and about two-thirds the distance to the head of the epididymis must be performed. This is achieved by first pulling the epididymis gently away from the testis and then removing overlying fatty tissue without destroying the small translucent ducts. The thin membrane around and the fibers between the ducts allow a small bundle of ducts to be dissected free. The ducts are cranial to the vascular pedicle, and dissection is most easily performed with the testis reflected laterally as shown in Figure 5A. The tip of the micropipette is inserted into a large duct at approximately the mid point between the testis and the head of the epididymis (Fig. 3, Pipette B). The wall of the duct is more resistant than the seminiferous tubule and penetration by the pipette tip is difficult. Entry is facilitated by using a sharp, beveled pipette tip and grasping the ducts and associated fibrous tissue surrounding the ducts close to the epididymis with fine forceps, thereby creating tension on the duct in order to stabilize it and provide resistance to the forward movement of the pipette. When the tip enters the duct, the pipette should be carefully threaded a short distance toward the testis (Fig. 5B). This decreases leakage of the cell suspension and stabilizes the pipette tip in the duct to prevent accidental removal. Movement of the operating field and particularly the testis should now be avoided since the fragile pipette tip can be broken in the duct. If this occurs, removal of the glass tip and cannulation of another duct is necessary.

The injection procedure through an efferent duct is shown stepwise in Figure 5. A bundle of ducts has been exposed and most of the fat removed (Fig. 5A). The delicate translucent ducts are difficult to photograph, but several are present in the bundle. In Figure 5B a large duct has been entered with a pipette tip, which is inserted almost to the rete. Following a pressure increase in the pipette, the cell suspension will flow first into the rete and then begin to fill the seminiferous tubules. However,

Fig. 5. Efferent duct injection. *In this technique, the micropipette is inserted into one of the large efferent ducts midway between the head of the epididymis and the testis.* **(A)** *The fatty tissue around the ducts has been removed. The ducts are difficult to visualize (arrow). A small amount of blue dye solution injected between the ducts under the fibrous sheath may help to outline the ducts.* **(B)** *A micropipette has been inserted into a large duct and threaded almost into the rete. In this position it is secure and fluid cannot leak around it. Pressure in the pipette has been increased and the rete filled with dye solution. Tubules are also beginning to fill.* **(C)** *The tubules on the surface are almost totally blue indicating that the solution has entered and filled most if not all areas of seminiferous tubules. Flow of solution into the rete has generally stopped at this point, and the other efferent ducts as well as some portion of the epididymis head will also contain the injected solution. Further increase in injection pressure is likely to result in testicular ischemia or rupture of the tunica. Dissection time was approximately 15 min, and tubule filling time was about 10 min.*

filling is not uniform in its spread from the rete, possibly because of variation in the resistance of tubules. The testis gradually fills with the cell suspension, and this may be facilitated by a slow increase in injection pressure. Care should be taken not to raise the pressure in the pipette high enough to cause blood to disappear from the testicular surface vessels or to rupture the tunica by a sudden surge in pressure. Surprisingly, the seal around the pipette becomes very tight and tunica rupture can easily result from excessive injection pressure; similarly, an ischemic testis can be produced. If ischemia occurs and does not disappear after several minutes, an incision in the tunica should be made to release pressure and reestablish blood flow. Frequently, a variable degree of adhesion will occur between exposed tubules and the surrounding fat pad, but this is preferable to returning the ischemic testis to the body cavity following injection. Very good filling occurs with this technique (Fig. 5C). Once mastered, surface tubule filling of 70 to 100% can be achieved in 15 to 30 min for both testes of an animal. Furthermore, only small volumes of donor cell suspension, between 100 and 150 µl are required, because little fluid is lost. This is demonstrated by the negligible amount of blue solution on the fat in Figure 5C compared to Figure 4C.

Rete injection

In order to inject donor cells directly into the rete testis, the testis is removed from the body cavity and reflected laterally. The rete lies primarily under the vascular pedicle where it contacts the surface. The most obvious vessels are the large veins that lie under the tunica and drain the testis. The primary arterial blood supply runs distal from the vascular pedicle on the dorsal surface, passes over the caudal pole and courses into the center of the testis on the ventral side of the organ. The most accessible portion of the rete is just cranial to the vascular pedicle (Fig. 6A). A small area of the rete extends out from under the vessels, and the efferent ducts arise at this point. Therefore, one can locate the surface of the rete by following the efferent ducts to the surface of the testis. In the mouse, the ducts enter the rete in a small area, which provides a landmark for initial identification. For injection, a sharp pipette with a tip diameter of 50 to 60 µm is inserted into the area cranial to the vessels and adjacent to the efferent ducts (Fig. 3, Pipette C). The pipette should be almost parallel (less than 30° angle) to the surface of the testis (Fig. 6B) since the depth of the rete is 0.2 mm or less. If the angle is too large or the forward movement of the pipette too great, the intratesticular boundary of the rete will

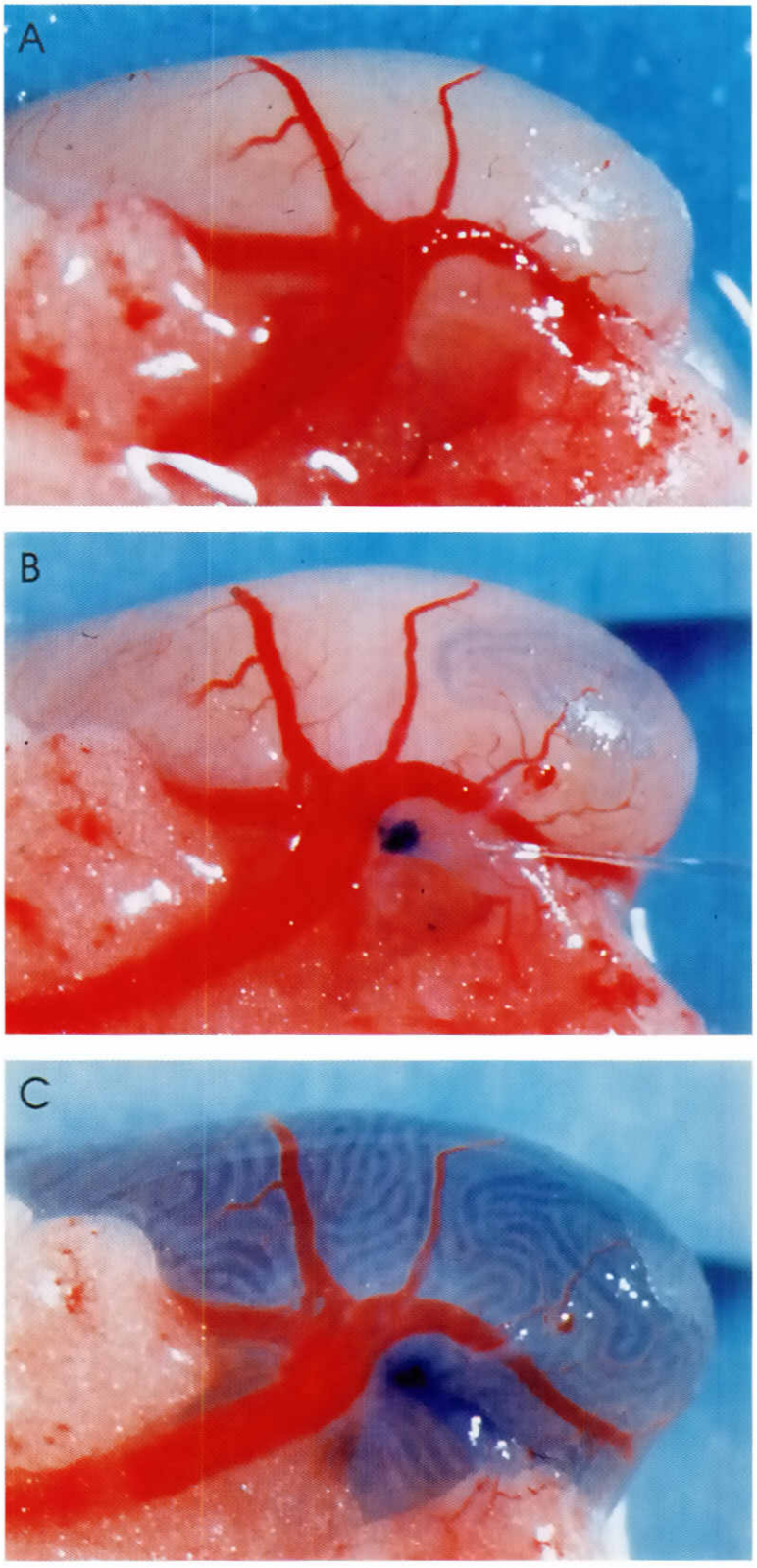
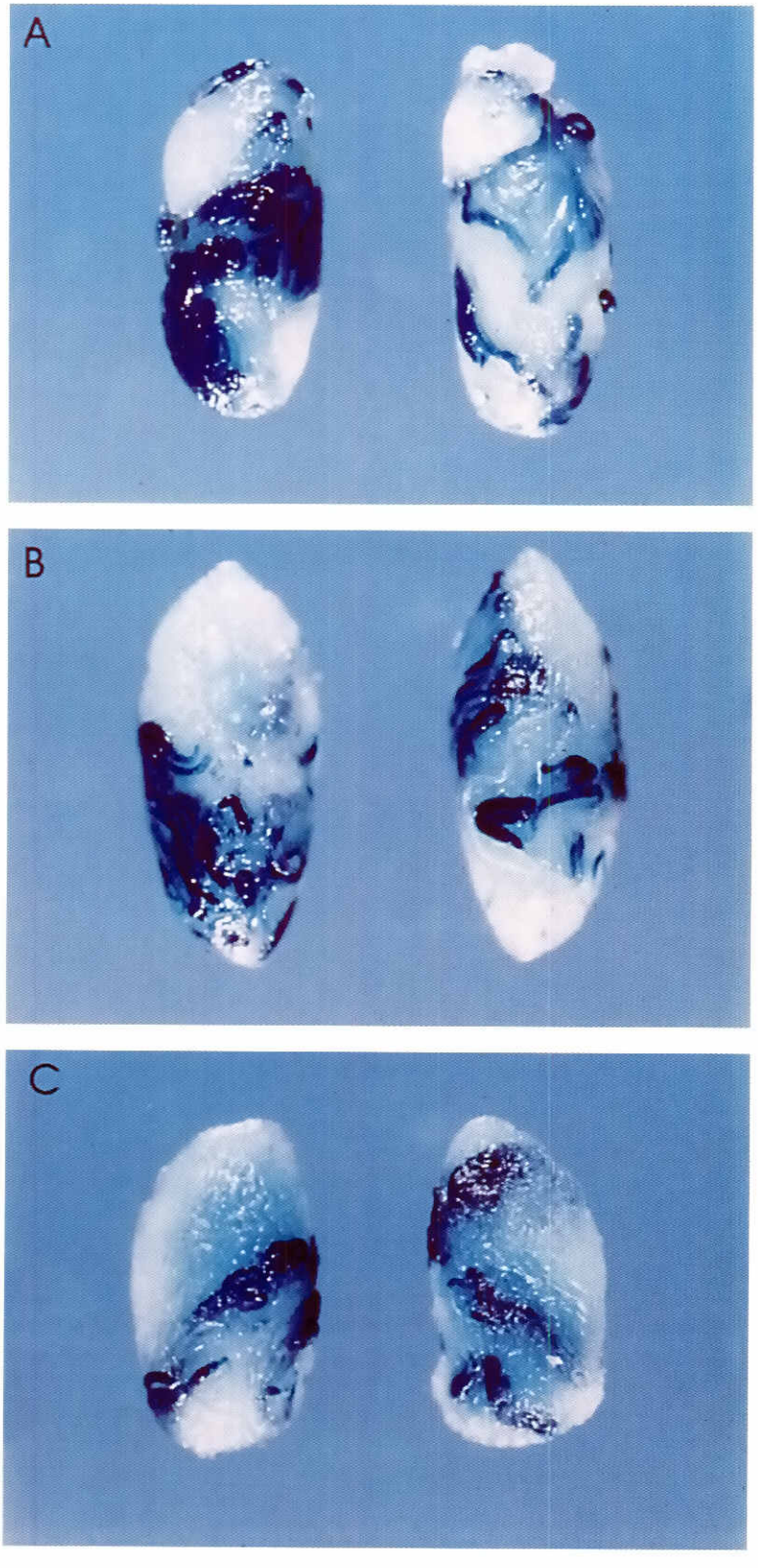


Fig. 6. Rete injection. *In this technique, the micropipette tip is inserted directly into the rete.* **(A)** *Surrounding fat and tissue has been carefully dissected away from the area cranial to the vascular pedicle connecting the testis to the testicular cord. The rete lies on the surface of the testis, mostly under the spreading branches of the vessels. The thick tunica covers most of the surface of the rete, but a small area extends cranial to the vessels and the tunica appears thinner in this area where the efferent ducts arise.* **(B)** *A micropipette has been inserted into the rete and a cell solution containing dye injected. The edge of the rete is now clearly visible and tubules have begun to fill.* **(C)** *The tubules of the testis are completely filled, and the pipette has been withdrawn. The cell injection of this testis was performed without a micromanipulator or pressure injector. The pipette was hand-held and pressure was created manually using a 1 ml syringe connected to the pipette with polyethylene tubing (see text). Dissection time was less than 10 min, and tubule filling time was less than 1 min.*



be penetrated, and the donor cell suspension will leak into the intertubular tissue rather than filling the tubules. Once the pipette tip has entered the rete, pressure in the injection tubing is increased until fluid fills the rete and flows into the tubules (Fig. 6B). Tubules fill in a random way similar to efferent duct injection. Pressure in the pipette should not be increased rapidly or raised too high, because leakage around the needle may occur or rupture of the internal rete boundary will result. Filling of the surface tubules is generally 50 to 100% (Fig. 6C) and takes 10 to 20 min for the two testes of a mouse. Care must be taken not to dislodge the tip from the rete because reentry into the same opening is usually difficult, and an additional penetration may not result in tubule filling since fluid leaks easily from the original entry point. An alternative approach to the rete is to introduce the pipette along and parallel to the efferent ducts within the fibrous bundle until it penetrates the rete. This technique will often allow one or two additional penetrations before leakage occurs. In a successful rete injection only 100 to 150 μl of cell suspension is required to fill two testes.

Analysis of recipient mice

Recipient males were generally maintained for 70 to 140 days before analysis, which represents 2 to 4 times the period necessary for spermatogenesis in the mouse. For analysis, testes were removed and dissected free of fat. A small piece of epididymis was included, separate from the testis, in the assay procedure to establish a positive staining control since epididymis has endogenous ß-galactosidase activity. The testes were fixed 1 h in 2 ml of 4% paraformaldehyde on ice with gentle rocking, bisected, and fixed for another hour. They were then washed with 3 changes of buffer at 30 to 60 min intervals and stained with X-gal as previously described (Brinster and Avarbock, 1994; Zambrowicz *et al.*, 1994). Each testis was examined at 12 to 50-fold magnification for the presence of tubules that stain blue. The histological appearance of some testes was examined. In these instances, 5 μm sections were cut and stained with neutral fast red. The number of tubule cross sections that stained blue was generally greater than the number of tubules visibly blue on the surface because of the tortuous path of the tubules within the testis.

Fig. 7. Colonization of recipient testes in busulfan treated mice following donor cell injection. *Blue staining of seminiferous tubules reflects colonization by transplanted spermatogonial stem cells which contained a LacZ transgene expressed only in round spermatids and later stages of spermatogenesis. Areas of seminiferous tubules in which there is no spermatogenesis or in which endogenous spermatogenesis may have regenerated will not stain blue.* **(A)** *The recipient mouse had been injected using the seminiferous tubule technique and was analyzed approximately 90 days later. A significant number of tubules are stained in both testes. The length of the stained segment varies among tubules, and the stained tubules are randomly distributed over the surface and within the testes. The rete is visible on the upper right surface of the right testis and appears as an area of radiating tubules.* **(B)** *The recipient mouse had been injected using the efferent duct as the injection site and was analyzed approximately 110 days later. The degree and random character of colonization is similar to that seen for seminiferous tubule injection.* **(C)** *The recipient mouse was injected into the rete and was analyzed approximately 150 days later. The degree and pattern of colonization is similar to that seen for the other two techniques.*

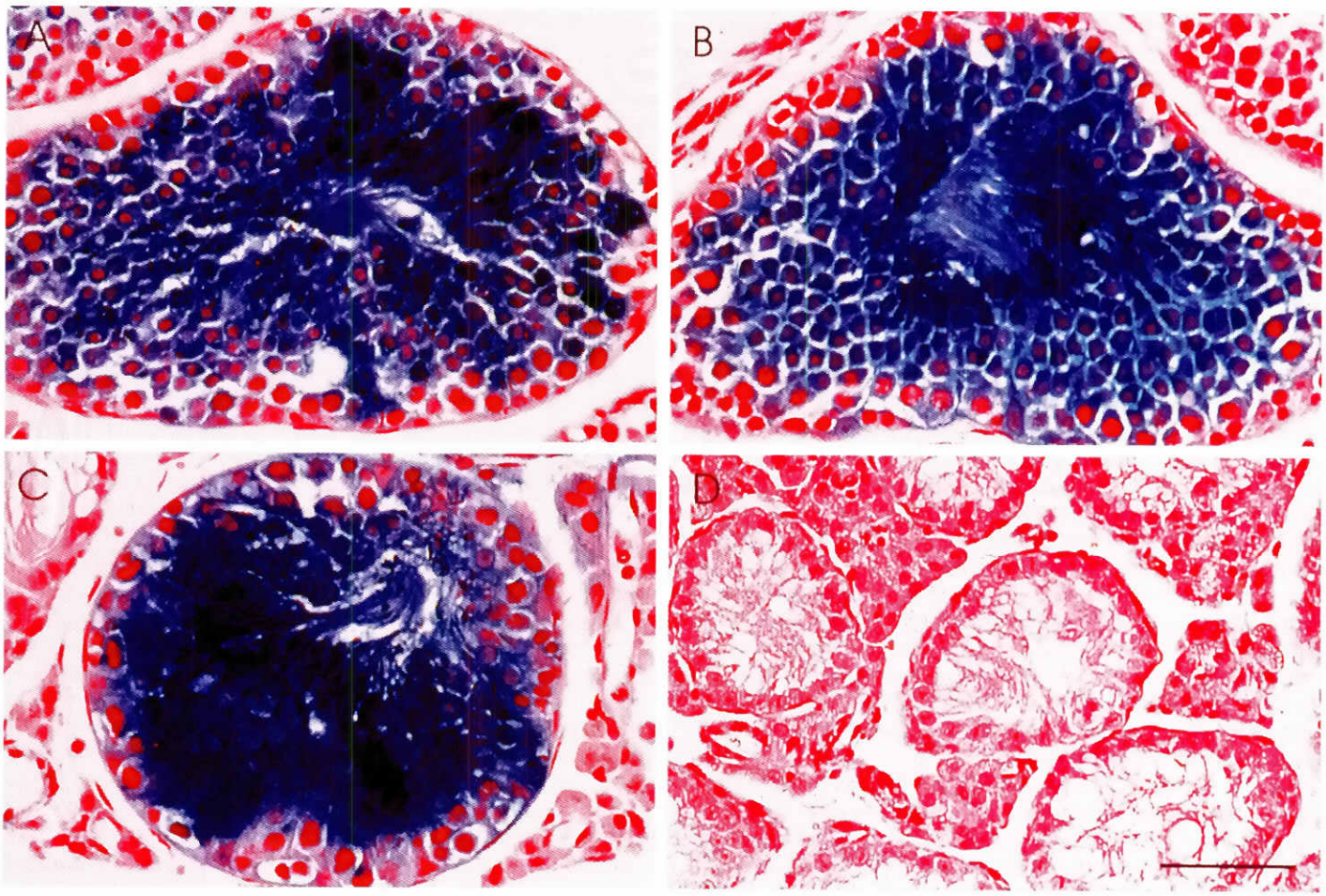


Fig. 8. Microscopic structure of spermatogenesis in recipient seminiferous tubules following donor testis cell transplantation by different techniques. (A) *The photomicrograph shows normal appearing spermatogenesis occurring from donor cells injected through the seminiferous tubule. Cross section is from the testes in Figure 7A.* **(B)** *The photomicrograph shows normal appearing spermatogenesis following donor cell injection into an efferent duct. The cross section is from the testes in Figure 7B.* **(C)** *The photomicrograph shows normal appearing spermatogenesis occurring from injection of donor cells into the rete. Cross section is from the testes in Figure 7C. The microscopic appearance of spermatogenesis arising from the three transplantation techniques is similar.* **(D)** *Typical appearance of the seminiferous tubules in a recipient mouse that did not receive testis cell transplantation (from Avarbock et al., 1996). The tubules contain only Sertoli cells. Blue color of cells in Panel A, B and C is from ß-galactosidase activity produced by the transgene in the donor cells. Transgene expression occurs only in round spermatids and later stages of germ cells. No somatic cells stain. Blue color in more immature stages of germ cells occurs by leaching of the stain into adjacent cells when activity is high. Cross sections of tubules with no spermatogenesis or regenerated endogenous spermatogenesis will not stain. Background stain in all sections is neutral fast red. Bar, 50 μm.*

Results and Discussion

Introduction of donor cells into the testes of recipient mice by any of the techniques described results in colonization of the seminiferous tubules and spermatogenesis. When donor cells are harvested from the testes of ZF*LacZ* mice, the areas of spermatogenesis arising from transplanted cells can readily be identified by incubating the testes with the substrate X-gal. Round spermatids and subsequent stages of germ cell differentiation will stain intense blue. Other cells in the testis, including any endogenous germ cells, will not stain. However, with the intense staining that results from excellent donor cell-derived spermatogenesis, leaching of blue color into spermatocytes and spermatogonia frequently occurs. If analysis of recipient testes is made at an interval longer than 35 days following donor cell microinjection, all blue tubules in the testes must arise from donor stem cell expansion and differentiation. Any injected partially differentiated donor cells should have completed differentiation or died within 35 days. Therefore, recipient mice are generally analyzed between two and five months following cell transplantation, which allows sufficient time for stem cell expansion and germ cell differentiation.

Examples of recipient testes microinjected by the three techniques described and analyzed for donor cell transplantation are shown in Figure 7. Each of the techniques produced good colonization of recipient testes, and in the best instances more than 12 tubules were stained in an individual testis. However, each of the techniques was characterized by a wide range in the number of tubules colonized, and the degree of spermatogenesis was not related to the method of introducing donor cells into the tubules of the testis. Microscopic examination of recipient testes demonstrated that normal appearing spermatogenesis occurred regardless of the technique used for donor cell introduction (Fig. 8). Thus,

the degree and quality of donor cell-derived spermatogenesis following each of the techniques appeared similar.

The anatomical structure of the testis consists of a group of seminiferous tubules connected at both ends to a common collecting area known as the rete testis, from which arise the efferent ducts that carry spermatozoa to the epididymis (Dym, 1983; Moore, 1995). While these ducts and tubules are interconnected, they have narrow lumens, and the total length of the tubular structures involved is large. Furthermore, anatomical constrictions have been described at the ends of each seminiferous tubule, which may act under some circumstances as valves to reduce or prevent flow of spermatozoa or fluid from the rete back into the tubules (Roosen-Runge, 1961; Hinton and Setchell, 1993). Previous studies have demonstrated that injected donor cells will colonize the seminiferous tubules of a recipient mouse (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994). In addition, it was reported recently that intraluminal spermatogenesis occurred in rat seminiferous tubules following injection of donor testis cells into the rete (Jiang and Short, 1995). However, the general effectiveness of donor cell injection into the rete or efferent ducts relative to direct injection into seminiferous tubules was unknown. The results reported here clearly establish that total filling of the surface tubules of the testis can be achieved following each of the three approaches. In view of the length and narrow lumen of the network, it could be argued that the appearance of the testis surface reflects only or primarily the presence of dye solution and that passage of the donor cells was restricted by bends, friction or functional impedance of tubular structures (Roosen-Runge, 1961; Free and Jaffee, 1979; Dym, 1983). That this is unlikely is shown by the significant colonization of recipient tubules by all three techniques. The random distribution of colonized sections of the tubules following injection by each of the techniques and the similar high level of colonization attainable with each approach suggests that they are all reasonable methods to distribute potential donor stem cells throughout the tubules of a recipient testis.

While all three approaches described have been successful in achieving similar levels of donor cell colonization, each has advantages and disadvantages that may make it most suitable for a particular experimental application. Direct injection of the tubules is simplest and allows many opportunities to enter different tubules at various sites on the testis surface. Thus, it may be a reasonable initial method for new investigators in the area. However, this approach can be the most time consuming, because several incisions of the tunica may be required with a corresponding number of pipette alignments and insertions into individual tubules. Furthermore, it is likely to be inappropriate and ineffective for larger testes, such as those of rats or domestic animals, where the number of tubules and length of each is greater than in the mouse. Injection of donor cells suspension through the efferent ducts is a more difficult technique to master initially and requires careful dissection to expose the delicate ducts. In addition, excessive internal testicular pressure can be induced by this technique which may create ischemia and interfere with donor cell viability as well as endogenous tubular environment. However, once the technique is mastered, it is faster than tubule injection and more reliable than rete injection. The pipette is held securely in the duct which results in little cell leakage. Thus, smaller quantities of cell suspension are needed to fill the tubules, which generally allows a higher concentration of donor cells in the injection solution. Furthermore, in the event of accidental withdrawal, the pipette can be reinserted into the original opening or another duct injected. Perhaps most important, the efferent ducts of most or all species should be accessible for injection. Injection into the rete requires less dissection than for efferent duct injection and is thus quicker. However, generally only one or two penetrations can be made before leakage prevents filling of the tubules. In addition to the speed of this approach, it is the most easily performed without the aid of a micromanipulator or pressure injector. While this approach is feasible in the mouse and rat, where the rete is superficial, it may be considerably more difficult in some species, such as monkey, pig or sheep, which have an axial rete deeply located in the testis and consisting of a network of channels (Dym, 1983). As experience increases with these three approaches, it is likely that each will find a useful place in various types of experiments to transplant donor cells to recipient testes of different species.

Although a high degree of colonization can be achieved with the three injection approaches, each is characterized by considerable variation in the area and distribution of donor cell spermatogenesis. Several factors appear related to this phenomena. Obviously, the degree to which the injected cell solution fills the tubules will influence the outcome, and surface tubule filling is the best criterion of this parameter. The convoluted nature of the tubules, including their course both on the surface and interior of the testis, suggests that surface coverage is a good representation of total tubule filling (Clermont and Huckins, 1961). It is difficult to predict the variation that may occur in seminiferous tubule environment and how this may affect colonization. However, within an experiment it is possible to use recipients of identical genetic background that have been prepared (e.g. by chemical treatment or irradiation) in the same manner and at the same time. This should minimize recipient variability. Probably the most important factor affecting the success of colonization is the donor cell population, specifically the number of cells capable of acting as spermatogonial stem cells within the injected suspension. The definition of a stem cell is based on functional capability, in particular, its ability to generate a colony of spermatogenesis (Meistrich and van Beek, 1993). Thus, it is impossible to identify the presence of a stem cell in a cell population by any morphological or chemical criteria currently available. Therefore, the potential of any cell population to colonize the seminiferous tubules of a recipient is difficult to quantify or estimate before injection. It has been suggested that there are approximately 10^8 cells in a mouse testis, of which about $2x10^4$ are estimated to be stem cells, or about 2 stem cells per 10^4 cells (Meistrich and van Beek, 1993; Tegelenbosch and de Rooij, 1993). If a donor testis cell population of $50x10^6$ cells per ml is used and the seminiferous tubules of a mouse testis have a total injectable internal volume of about 10 µl (Brinster and Avarbock, 1994 and our unpublished data), then approximately 100 stem cells would be introduced following a good injection procedure. Our previous reports (Brinster and Avarbock, 1994; Avarbock *et al.*, 1996; Clouthier *et al.*, 1996) and unpublished observations suggest that 10 to 40 focal areas of donor cell-derived spermatogenesis within recipient tubules often can be identified by blue staining. This would represent an efficiency of 10 to 40%. However, these calculations are quite conjectural, since the number of stem cells is unknown, and the number of blue stained tubules is difficult to count and each stained area varies in length. It is unknown whether a length of blue tubule represents the expansion of a single stem cell or the merging

of several stem cell colonies. As techniques to identify potential populations of stem cells are developed, the spermatogonial transplantation technique can be used to verify the colonizing potential of these putative stem cells. In this way, criteria to identify spermatogonial stem cells can be developed, which will allow quantitative evaluation of spermatogenesis and detailed studies of factors affecting this complex and essential process.

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