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Measurement of Immunoglobulin G Levels in Adult Rat Testicular Interstitial Fluid and Serum

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ABSTRACT: Total immunoglobulin G (IgG) in rat testicular interstitial fluid and serum was measured by two-site enzyme-linked immunosorbent assay, which displayed minimal cross-reactivity with IgA or IgM. Normal adult testicular fluid IgG concentrations (2.67 ± 0.98 mg/ml) were found to be 65% of the levels found in serum. Disruption of seminiferous tubule function by experimental cryptorchidism had no effect on either testicular or serum IgG concentrations. Castration also had no effect on serum IgG concentrations. The effect of the Leydig cell cytotoxin, ethane dimethane sulfonate, on IgG concentrations was obscured by the observation that dimethyl sulfoxide (the carrier solvent) itself effectively eliminated the IgG concentration gradient across the testicular microvasculature. In normal and cryptorchid rats, serum IgG and thymus weights showed no correlation with serum inhibin concentrations measured by radioimmunoassay, although thymus weights were significantly increased in castrate rats. The data confirm that the permeability of the rat testis to circulating IgG is relatively high compared with other tissues and suggest that the testicular transendothelial transfer of IgG is unaffected by changes in testicular activity or hormone secretion. The high intratesticular concentration of IgG and its apparent lack of local regulation provide further evidence that the phenomenon of immune privilege in this tissue cannot be attributed simply to restriction of immune effectors from the testicular environment. The data also indicate that serum IgG levels in the adult male rat are not influenced in the short-term by changes in peripheral testicular androgen levels. The direct effect of dimethyl sulfoxide on testicular endothelial permeability requires further investigation, as this solvent is used as a carrier for nonaqueous reagents in many studies of testicular function *in vivo*.

Key words: Testosterone, inhibin, immune system, dimethyl sulfoxide, ethane dimethane sulfonate, enzyme-linked immunosorbent assay.

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The rat testis interstitium is an immunologically privileged site for tissue grafts (Head et al, 1983). It has been demonstrated that this privilege is not due to restriction of immune effector cells (Niemi et al, 1986), and that circulating antibodies have access to the testis of all species studied (Johnson and Setchell, 1968; Koskimies et al, 1971; Beh et al, 1974; Alexander and Anderson, 1987; Yule et al, 1988). In fact, vascular permeability to circulating immunoglobulin G (IgG) increases markedly at puberty in the rat testis apparently in conjunction with an overall increase in permeability to all serum proteins (Pöllänen and Setchell, 1989).

Previous studies have indicated that testicular hormones, particularly androgens and inhibin, influence peripheral immune cell functions *in vivo* and *in vitro* (Grossman, 1989; Hedger et al, 1989). It also has been shown that modulation of Leydig cell and germ cell numbers has a direct effect on vascular fluid permeability characteristics in the rat testis (Sharpe, 1983; Damber et al, 1985, 1987; Maddocks and Sharpe, 1989). Consequently, alterations in testicular function and circulating hormones may be expected to influence both serum and testicular interstitial fluid (IF) IgG concentrations.

The concentration of IgG in either extravascular IF or lymph, which is directly related to the organ vascular permeability of the protein, ranges from 9% to 28% of blood levels in several nontesticular rat tissues (Auckland et al, 1984; O'Connor and Bale, 1984). However, the relative concentration of IgG in IF from the rat testis has not been assessed previously. An earlier study of IgG in sheep testis lymph indicated lymph : serum ratios of 54% and 63% for the two ovine IgG subclasses, which were similar to ratios found in other tissues of the sheep (Beh et al, 1974). In contrast to the rat, however, the sheep testis does not appear to be immunologically privileged (Maddocks and Setchell, 1988) and has a slightly faster lymphatic flow rate (Setchell, 1986). Moreover, the rat testis lymphatic endothelium, unlike that found in the sheep testis, is incomplete allowing free exchange between the testicular IF and lymph (Fawcett et al, 1973). Altogether, these differences suggest that immunological parameter data obtained from studies of the sheep testis

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may not be indicative of the rat testis environment. In the case of nontesticular tissues, lymph-to-blood IgG concentration ratios in the rat appear to be much lower than those of the same tissues of the sheep (Beh et al, 1974; Mullins et al, 1991). On the other hand, the apparent IgG permeability–surface area product of the rat testis vasculature (Pöllänen and Setchell, 1989) appears to be severalfold higher than that of other nontesticular tissues in the rat (Renkin et al, 1993).

In the following study, an enzyme-linked immunosorbent assay (ELISA) specific for IgG was developed in order to measure the testicular IF and serum concentrations of total IgG in normal adult rats. These parameters were also measured in rats with spermatogenic disruption induced by experimental cryptorchidism, or ablation of the Leydig cells by treatment with ethane dimethane sulfonate (EDS) in order to determine whether testicular IgG concentrations are regulated locally.

Materials and Methods

Animals

Adult male Sprague-Dawley rats (80–100 days old) were obtained from the Monash Central Animal House.

Experimental Details

Adult rats (n = 9) were ether-anesthetized and a sample (1–2 ml) of blood was collected by cardiac puncture. The blood was allowed to clot (4 hours, 20°C) and the serum fraction collected. The testes were removed and one testis was perfused, via the capsular artery, with Dulbecco's phosphate-buffered saline, pH 7.4, using a peristaltic pump (0.2 ml/minute) to remove blood from the vascular bed. Perfusion continued until all blood was cleared from the surface vessels (usually less than 1 minute). Testicular IF was collected from a 2-mm incision in the lower pole of the testis, as previously described (Hedger et al, 1990). All samples were stored at −20°C until assay.

Adult rats were castrated or made either unilaterally or bilaterally cryptorchid by transferring one or both testes into the abdominal cavity, sectioning the gubernaculum, and closing the inguinal canal with a suture (Risbridger et al, 1981), 4 weeks prior to collection of samples. Cryptorchidism in adult rats induces a rapid loss of the spermatogenic cells from the seminiferous epithelium and alteration in Sertoli cell morphology and function (Kerr et al, 1979a). At 4 weeks, the cryptorchid intratesticular testosterone concentration is considerably reduced, but serum testosterone levels remain within the normal range (Kerr et al, 1979b; Sharpe et al, 1986). Cryptorchid and castrate rats and their respective unoperated controls (n = 5 rats/group) were killed by decapitation. Trunk blood serum and testicular IF and serum were collected as described above. At the same time, thymus, spleen, seminal vesicles, and prostate were dissected free of fat and weighed.

Samples of testicular IF and serum from adult rats in which the Leydig cell population had been ablated by EDS treatment for either 10 days or 41 days were obtained from a previous experiment (Wang et al, 1994). Briefly, adult male rats were randomly assigned to one of the following experimental treatments and received: (1) no treatment, (2) a single injection intraperitoneally (i.p.) of 25% dimethyl sulfoxide (DMSO) in water (200 μl/100 g body weight), (3) a single injection (i.p.) of EDS to a final dose of 7.5 mg/100 g body weight dissolved in 25% DMSO: water (200 μl/100 g body weight), and (4) a 3-cm testosterone-filled silastic capsule subcutaneously (s.c.) followed by a single injection of EDS. At 10 days after EDS administration, Leydig cells are completely absent from the testis, but the Leydig cell population and intratesticular testosterone levels are restored to normal within 41 days, by recruitment of unidentified precursor cells. A 3-cm testosterone implant maintains serum testosterone levels, while suppressing Leydig cell recovery and preventing restoration of normal intratesticular testosterone levels. Testicular IF and serum (by cardiac puncture) were collected, as described above.

Immunoglobulin G ELISA

Total serum and IF IgG was measured by a two-site ELISA employing a sheep anti-rat IgG (Silenus Laboratories, Victoria, Australia) as capture antibody (1:800) in Greiner 96-well assay plates (Greiner Labortechnik, Frickenhausen, Austria), purified rat serum IgG (Sigma Chemical Co., St. Louis, Missouri) as reference standard (0.01–2.5 μg/ml), and a rabbit anti-rat Ig conjugated to horseradish peroxidase (DAKO A/S, Glostrup, Denmark) as secondary antibody (1:4,000). Samples (serum and testicular IF) were assayed in duplicate at multiple dilutions for standard bioassay analysis, with appropriate sample blanks. The chromogenic reagent, o-phenylenediamine (0.2%) in citrate-phosphate buffer, pH 5.2, with 0.02% H_2O_2, was added for 15 minutes, and the reaction halted by addition of H_2SO_4. Absorbance of the reaction product was measured in a Titertek Multiscan MC plate reader (Flow Laboratories, Maclean, Virginia) at 490 nm, and sample blank values (nonspecific absorbance) were subtracted. Assay characteristics were determined by assay of globulin-free rat serum albumin (Sigma), rat myeloma immunoglobulin subclasses (kappa) IgA, IgM, and IgG_{2a} (Serotec, Oxford, U.K.), and serum from mouse, rabbit, goat, and sheep (Silenus).

Inhibin Radioimmunoassay (RIA)

Immunoreactive inhibin levels in serum were measured by heterologous double-antibody RIA using a rat ovarian extract as standard, as previously described (Robertson et al, 1988).

Protein Assay

Proteins were measured by DC protein assay (Bio-Rad, Hercules, California), using bovine serum albumin as standard (Lowry et al, 1951).

Statistical Analysis

Relative potencies were obtained by comparisons of the ELISA standard and sample log-linear transformed dose–response curves over the effective linear response range (approximately 25–75% of the maximum OD_{490} response), using standard bioassay sta-

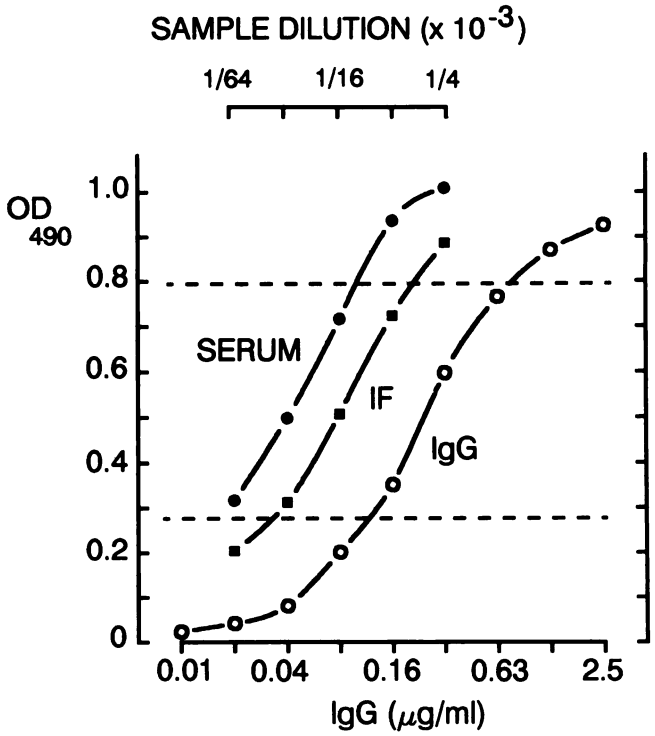

FIG. 1. Immunoglobulin G (IgG) ELISA log dose–response curves for IgG standard (○—○), serum (●—●), and testicular IF (■—■). Values are mean of duplicate determinations. ·····: upper (75% maximum assay OD_{490}) and lower (25% maximum assay OD_{490}) limits of linear log dose–response range used to determine bioassay statistics.

tistics (Finney, 1964). Comparisons between individual responses were made by paired *t*-test or by Peritz's multiple range test (Harper, 1984). The relationship between serum inhibin concentration and serum IgG concentration or thymus weight was determined by linear regression analysis.

Results

IgG ELISA Characterization

Both rat serum and testicular IF displayed linear log dose–responses in the ELISA that were parallel with the rat IgG standard within the approximate response range of 25–75% maximum OD_{490} (b = 0.244 ± 0.042; mean ± SD, n = 19 assays), covering a minimum of three doses (Fig. 1). Rat serum albumin (globulin-free) did not cross-react in the assay (<0.1%), nor did serum from mouse, sheep, rabbit or goat. The rat immunoglobulin subclasses IgA and IgM cross-reacted 1.4% and 0.4%, respectively, while IgG_{2a} (the major IgG subclass present in rat serum) cross-reacted 114%. The assay had a mean λ value of 0.05 and an interassay coefficient of variation of 17.3% (n = 19 assays).

Effect of Vascular Perfusion on IgG Levels in Testicular IF

The levels of IgG in testicular IF were 65% of those found in serum, compared with 89% for total protein (Table 1). Perfusion of the testis prior to collection caused no significant reduction in testicular IgG levels (either total, or calculated as mg IgG/mg protein). However, there was a small, but significant, increase in IF volume collected (32%) and a corresponding reduction in total protein concentration (24%), indicating a dilution of IF during or following perfusion (Table 1).

Effects of Cryptorchidism and Castration

Destruction of the seminiferous epithelium by cryptorchidism (4 weeks) had no effect on the ratio between serum and testicular IF IgG concentrations (Fig. 2A), although there was a twofold increase in mean testis IF volume at this time in the cryptorchid testes (204 ± 31.9 μl compared with 100 ± 16.3 μl; mean ± SD, n = 5 animals). In the same animals, the weights of the testes fell to 32.7% of control levels (0.56 ± 0.06 g compared with 1.71 ± 0.15 g mean testis weight; mean ± SD). Neither cryptorchidism nor castration (4 weeks) had any effect on serum IgG levels (Fig. 2A).

There was a significant increase (twofold) in thymus weight (Fig. 2B) and a corresponding decrease (sixfold) in seminal vesicle weight (Fig. 2C) in the castrate group. There was no change, however, in thymus or seminal vesicle weight in the bilaterally cryptorchid (Fig. 2B,C) or unilaterally cryptorchid (data not shown) rats. These observations were consistent with the anticipated fall in circulating testosterone levels in the castrate rats, and maintenance of normal testosterone levels in the cryptorchid rats. There was no correlation between either serum IgG

Table 1. *Evaluation of serum IgG levels in normal adult rats and IgG levels in IF collected from testes with and without prior perfusion with Dulbecco's phosphate-buffered saline**

	Volume (μl)	IgG (mg/ml)	Total protein (mg/ml)	mg IgG/mg protein)
Serum	—	4.11 ± 1.56	71.8 ± 11.2	0.059 ± 0.027
Interstitial fluid (unperfused)	61.3 ± 24.7	2.67 ± 0.98†	64.6 ± 6.9^{NS}	0.042 ± 0.015^{NS}
Interstitial fluid (perfused)	93.7 ± 37.1‡	2.45 ± 1.05^{ns}	49.3 ± 6.7‡	0.049 ± 0.021^{ns}

* Values are mean ± SD, n = 9 animals. Comparisons are between serum and unperfused testis (†P < 0.01; ^{NS} not significantly different) or between unperfused and perfused testis (‡P < 0.05; ^{ns} not significantly different) by paired *t*-test.

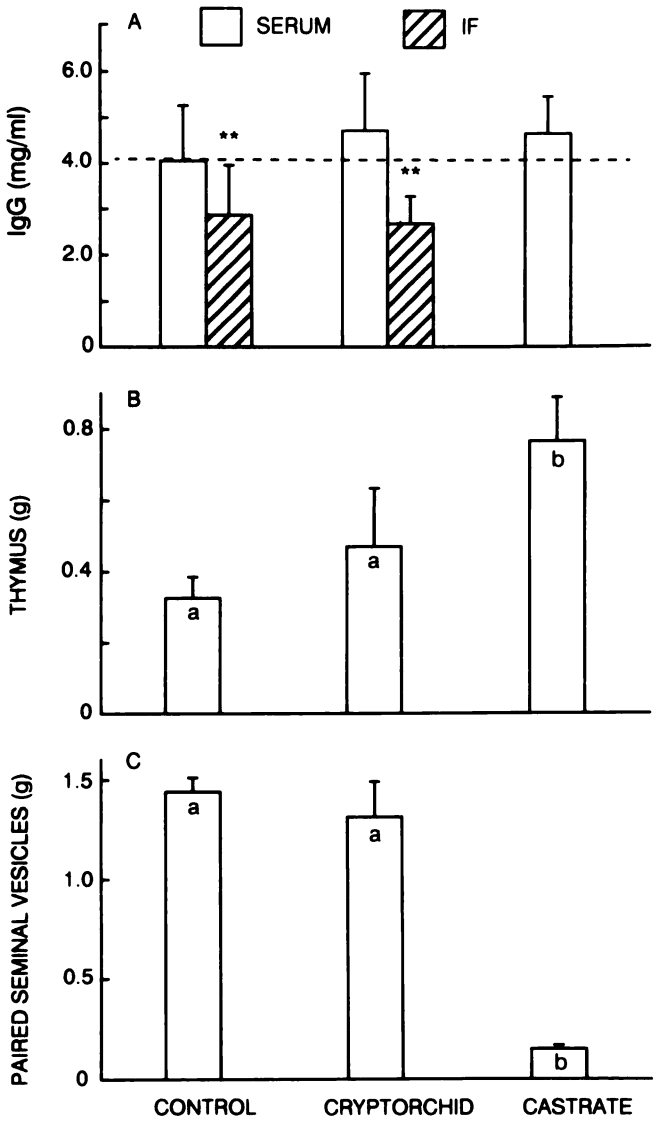

FIG. 2. Serum (□) and testicular IF (▩) IgG levels (A), thymus weights (B), and seminal vesicle weights (C) in bilaterally cryptorchid and castrated adult rats at 4 weeks after treatment. Values are mean ± SD, $n = 5$ animals/group. ** $P < 0.01$ comparison between serum and IF value by paired t-test. Values with same letter superscript are not significantly different by multiple range test ($P > 0.05$).

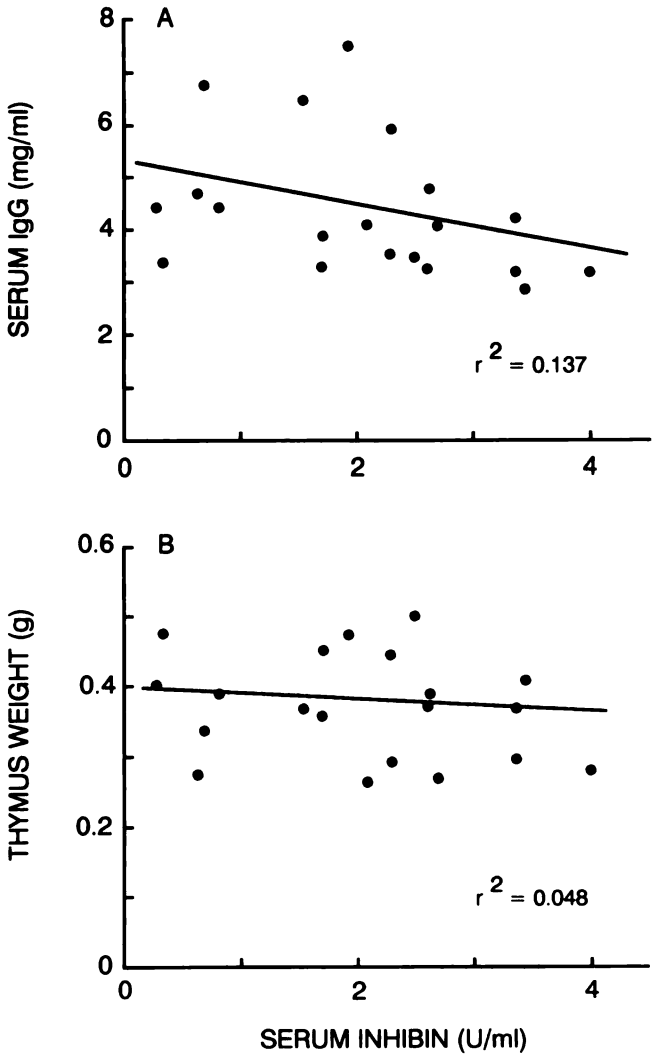

FIG. 3. Linear regression analysis of serum inhibin levels and serum IgG levels (A) and thymus weights (B) in untreated control and unilaterally or bilaterally cryptorchid adult rats.

concentrations, or thymus weights, and serum inhibin levels in the normal (2.92 ± 0.66 inhibin U/ml; mean ± SD), bilaterally cryptorchid (0.55 ± 0.23 inhibin U/ml), and unilaterally cryptorchid (2.18 ± 1.03 inhibin U/ml) groups (Fig. 3).

Effects of DMSO, EDS and Testosterone Implants

At 10 days after treatment, there was a significant (30%) decrease in serum IgG concentration in the DMSO-treated animals but not in either of the EDS-treated groups (Fig. 4A). At the same time, there was no difference between serum and testicular IF IgG concentrations in all three experimental groups, i.e., the IgG concentration gradient between serum and testicular IF was eliminated in all animals receiving DMSO, regardless of the presence of EDS or exogenous testosterone.

The IgG concentration gradient between serum and IF had not been restored at 41 days after treatment in the DMSO control and EDS-treated/testosterone-implanted groups (Fig. 4B). By contrast, in the animals treated with EDS alone, a significant reduction in testis IgG levels compared with serum restored the gradient between serum and testicular IF IgG levels (IF:serum IgG ratio: 0.72 ± 0.20; mean ± SD, $n = 6$ animals). Although there were no significant differences among either the serum or the testicular IF mean IgG concentrations at day 41, all three experimental groups showed a larger animal-to-an-

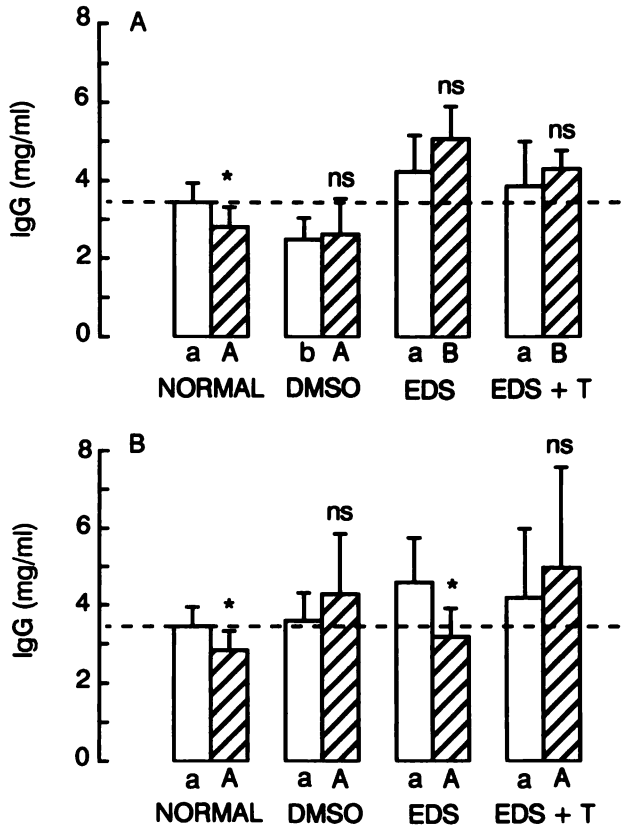

FIG. 4. Serum (□) and testicular IF (▨) IgG levels in adult rats at 10 days (A) and 41 days (B) after treatment with DMSO alone, EDS, or EDS with testosterone implants (EDS + T). Values are mean ± SD, $n = 6$ animals/group. * $P < 0.05$; ns $P > 0.05$ comparison between serum and IF value by paired t-test. Values with same letter are not significantly different by multiple range test ($P > 0.05$).

imal variation in serum and testicular IgG concentrations compared with the untreated controls (Fig. 4B).

Discussion

An ELISA specific for rat IgG was developed and used to establish that adult rat testicular IF contains a significantly lower IgG concentration than serum but maintains a considerably higher IF-to-serum IgG ratio than other rat tissues that have been measured previously (Auckland et al, 1984; O'Connor and Bale, 1984). Selective destruction of the seminiferous epithelium by experimental cryptorchidism and alterations in the levels of the testicular hormones testosterone and inhibin had no effect on either serum or testicular IgG concentrations. These observations support a previous report that the adult rat testis vascular endothelium has a high apparent permeability to IgG (Pöllänen and Setchell, 1989) and further suggest that peripheral IgG production and IgG access to the testis are not under testicular control in the adult rat. The apparent lack of restriction of IgG from the rat testis interstitium provides further support for the hypothesis that a local intratesticular immunosuppressive mechanism, rather than immune system exclusion, is responsible for the reduced immune responsiveness within this tissue.

Studies on the protein composition of rat testicular IF are usually complicated by problems of collection, particularly potential contamination from intracellular proteins (see review by Setchell, 1986). However, there are no B-lymphocytes in the rat testis (Wang et al, 1994) and consequently intratesticular IgG is unlikely to come from local production. The problem of serum contamination, either as a result of damage to capsule microvessels, or by transfer of blood-borne factors into the IF compartment during collection, is also a potential issue with the method employed. The use of saline to perfuse the testes in order to reduce potential serum contamination during collection caused a small, but significant, dilution of fluid (25–35%). The perfusion flow rate employed corresponded to the flow rate of blood under normal conditions, although some testicular distension was noted, suggesting that the lower osmolarity and/or nonphysiological flow characteristics may have been responsible for increased transfer of fluid into IF (Widmark et al, 1986; Renkin et al, 1993). When IgG concentration was corrected for this dilution, however, there was no significant difference in IgG levels, indicating that the contribution from serum during collection in unperfused testes was negligible. While these data indicated that it was unnecessary to perfuse the testis to remove serum prior to measurement of IgG, other serum proteins present at lower concentrations in testicular IF may still represent a problem.

The concentration of serum IgG in IF or lymph is dependent upon the permeability of the vascular endothelium to IgG and is inversely related to the rate of lymph flow (Garlick and Renkin, 1970). Although lymph flow was not measured in the present study, normal rat testis lymphatic drainage rates are comparable to those observed in other tissues (Aukland et al, 1984; Setchell, 1986; Renkin et al, 1993). While extracellular matrix charge exclusion characteristics and uptake by local cells such as macrophages also could affect IgG levels in the interstitial tissue (Aukland et al, 1984; Pöllänen and Setchell, 1989), these influences would tend to reduce IgG concentrations in the collected testicular IF. Consequently, it is reasonable to conclude that the relatively high levels of IgG in the testis are due to a relatively high apparent permeability of the testicular vascular endothelium compared with other tissues (Pöllänen and Setchell, 1989; Renkin et al, 1993). The large IF-to-serum IgG ratio in the rat testis in comparison with other rat tissues contrasts with earlier studies in the sheep, where the testis lymph : serum IgG ratio was found to be similar to that

of other tissues (Beh et al, 1974; Mullins et al, 1991). Whether these differences have any bearing on the reason why the sheep testis, unlike the rodent testis, does not appear to be a privileged site for grafts remains to be investigated.

Testosterone reportedly inhibits T- and B-lymphocyte functions, including immunoglobulin synthesis and secretion, *in vivo* and *in vitro* (see review by Grossman, 1989), and inhibin stimulates T-lymphocyte proliferation *in vitro* (Hedger et al, 1989). However, manipulation of circulating inhibin, by experimental cryptorchidism, and testosterone, by castration, had no direct effect on serum IgG levels in the present study. Consequently, total serum IgG levels appear to be unaffected by changes in the major circulating testicular hormones, testosterone and inhibin, in the adult rat at least in the short term. In contrast, those treatments that altered circulating testosterone levels (castration and EDS treatment) caused a significant increase in thymus weight, confirming a direct effect of testosterone on T-lymphocyte development, as previously observed (Grossman, 1989; Wang et al, 1994).

The only treatment to affect serum IgG levels in the present study was DMSO administration, which caused an acute reduction in IgG 10 days later. It is well known that chronic, or high-dose, administration of DMSO has immunosuppressive effects on B-lymphocyte function and antibody responses *in vivo* (Weetmen et al, 1982; Pestronk et al, 1985), and it appears that even the relatively small dose employed in the present study can have a significant effect on these functions. A long-term perturbation in B-lymphocyte function also was indicated by the large variation in serum and testicular IgG levels in the DMSO-treated animals groups, but not in untreated control rats, at 41 days after treatment. Interestingly, the initial reduction in serum IgG at 10 days was not seen in either of the EDS-treated groups, raising the possibility that EDS directly antagonizes the acute DMSO action on immunoglobulin secretion *in vivo,* suggesting a novel action of EDS outside the male reproductive tract.

Several previous studies have indicated that IF volume and, consequently, vascular fluid permeability in the testis is under the control of local factors, such that IF volume decreases upon androgen withdrawal and increases following depletion of the developing germ cells (Sharpe, 1983; Damber et al, 1985, 1987; Maddocks and Sharpe, 1989; Sharpe et al, 1991). Similar IF volume changes are reported in the current study and in the previous report (Wang et al, 1994). In contrast, there was no evidence in the current study that access of IgG to the testis is under local control. The testicular IgG concentration gradient appeared to be largely unaffected by depletion of the seminiferous epithelium following experimental cryptorchidism. Unfortunately, any effect of Leydig cell removal by EDS treatment on testicular IgG levels was obscured by the observation that DMSO alone effectively eliminated the concentration gradient across the testicular vascular endothelium. This loss of gradient did not appear to be attributable to an interruption of testicular lymph flow, as the testicular IF volumes in the DMSO-treated and EDS-treated groups were similar to, or less than, those of the untreated control group (Wang et al, 1994), consistent with previous observations after EDS treatment (Damber et al, 1987; Maddocks and Sharpe, 1989). Consequently, the data indicate an effectively free permeability of the testicular vascular endothelium to IgG in all DMSO-treated rats, and this complete permeability to IgG appears to be uninfluenced by the presence or absence of the Leydig cells. Moreover, these data indicate that transport of IgG into the rat testis is not linked to changes in vascular fluid permeability.

The data concerning permeability of the testicular capillaries to serum proteins tend to be inconclusive in relation to the mechanisms involved. The testicular capillary endothelium is unfenestrated, and it appears most likely that access to the testis by serum proteins, including IgG, principally involves endocytic transport across the endothelium (Taylor and Granger, 1984), particularly as several much smaller molecules are restricted (Kormano, 1967, 1968; Weihe et al, 1979). Although many serum proteins are found in testicular interstitial fluid and lymph, a recent ultrastructural and imunohistochemical study has suggested that the rat testis capillaries possess many characteristics of a highly selective endothelium, such as that found in the blood–brain barrier (Holash et al, 1993). DMSO reportedly alters the membrane properties of cells and tissues to increase molecular permeability and has been shown to increase the transendothelial transport of marker proteins through the blood–brain barrier, although the actual mechanism of action is not known (Brink and Stein, 1967; Balin et al, 1987). The results of the present study indicate that DMSO apparently has a similar effect on protein, or at least IgG, transendothelial transport in the testis. However, reported DMSO effects on the vascular endothelium are complex, as this solvent also antagonizes fluid permeability increases and edema due to its ability to inhibit both free-radical formation and vascular leukocyte adherence (Sekizuka et al, 1989; Wilson, 1990). What was surprising in the present study is the observation that the apparent IgG permeability increase was still evident in some treatment groups 41 days after the first injection, suggesting a prolonged alteration in the endothelial cell function of the testis. The recovery of the gradient in only the EDS-treated group suggests that growth factors associated with Leydig cell repopulation may have an influence on recovery of endothelial IgG permeability characteristics after DMSO treatment.

The effects of DMSO on serum and testicular IF levels of IgG was consistent with the earlier observation that

DMSO alone transiently (3–10 days after treatment) increased testicular T-lymphocyte traffic in the same experiment (Wang et al, 1994). Clearly, DMSO has significant effects on testicular endothelial protein and fluid permeability and the local immune system, which requires further investigation, particularly as DMSO is widely used as a carrier for nonaqueous reagents in studies of testicular function *in vivo*.

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