Journal of Andrology, Vol. 17, No. 2, March/April 1996
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Detection of Germ Cell-Derived Proteins in Testicular Interstitial Fluid: Potential for Monitoring Spermatogenesis *In Vivo*

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ABSTRACT: The aim of the present study was to assess whether proteins secreted by the seminiferous tubules (ST) can be detected in testicular interstitial fluid (IF) and testicular (TV), spermatic (SV), and peripheral venous (PV) plasma from adult rats. An antiserum was raised against seminiferous tubule-conditioned medium (STCM) prepared from adult rats and used in conjunction with Western blot analysis to screen IF and blood samples resolved by one-dimensional (1-D) and two-dimensional (2-D) sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Samples of IF and PV were analyzed from control adult rats and rats exposed to scrotal heating (43°C for 30 minutes) 24 hours earlier to ascertain whether damage to spermatogenesis would affect 'leakage' of proteins from the seminiferous tubules. In all control rats, the STCM antiserum specifically detected three proteins in testicular IF with molecular weights of 24, 16, and 14 kDa, respectively. Heat treatment increased the abundance of these proteins and induced the appearance of several other less-abundant proteins, all with molecular masses below 25 kDa. Two of the proteins present in IF were identified, the 24-kDa protein as phosphatidylethanolamine-binding protein (PEBP), and the 14-kDa protein as an androgen-regulated protein (ARP-2). Both of these proteins have been shown in previous studies to be secreted by round spermatids. Our results suggest that germ cell secretory products can gain access to the interstitium under both normal physiological conditions and more easily after induction of damage to spermatogenesis. The antiserum was unable to detect any ST-derived proteins in blood, although it is likely that this result may be due to insensitivity of the presently used techniques. The development of specific immunoassays for germ cell-secreted proteins (e.g., PEBP and ARP-2) should enable more definitive assessment of whether proteins secreted by the seminiferous epithelium can be measured in blood and thus provide a potential means of monitoring spermatogenesis.

Key words: Seminiferous tubule-secreted proteins, phosphatidylethanolamine binding protein, ARP-2, spermatid.

J Androl 1996;17:127–136

Disorders of spermatogenesis are presently only detectable by changes in sperm parameters in the ejaculate or by infertility. When and where in spermatogenesis these defects arise is largely unknown because our understanding of this complex process is poor and there are no means (other than testicular biopsy) of monitoring events within the seminiferous tubules. The ideal would be to identify proteins that play key roles in spermatogenesis and that also find their way into the bloodstream, where measurement of their levels might provide important insight into the normality or otherwise of particular steps in spermatogenesis (Sharpe, 1992).

Studies in men have attempted to correlate the levels of Sertoli cell-secreted proteins in either seminal plasma or the peripheral circulation with male infertility. Transferrin levels in seminal plasma show a good correlation with sperm counts but are apparently unrelated to seminiferous tubule status (Sharpe, 1992). Serum inhibin levels have failed to show any straightforward relationship to testicular damage (de Kretser et al, 1989) even though inhibin secretion is affected by adverse changes to spermatogenesis (McLachlan et al, 1988; de Kretser and Robertson, 1989; Sharpe, 1993). Moreover, the detection and measurement of Sertoli cell-secreted proteins in blood is often problematical because they show a high degree of homology to proteins produced at extratesticular sites. For example, androgen-binding protein (ABP) is similar to sex hormone-binding globulin secreted by the liver (Cheng et al, 1984), testibumin is similar to serum albumin (Cheng and Bardin, 1986; Cheng et al, 1987), and sulfated glycoprotein-2 (SGP-2) shows homology to SP-40, an inhibitor of complement-induced cell lysis (Jenne and Tschopp, 1989).

The detection of germ-cell derived proteins in blood may avoid these drawbacks, because many appear to be

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Received for publication August 15, 1995; accepted for publication October 27, 1995.

unique to the testis (Sharpe, 1992). However, there is only limited evidence that these proteins gain access to the bloodstream. One example is the isoenzyme lactate dehydrogenase-C4 (LDH-C4), which is unique to pachytene spermatocytes, round and elongating spermatids, and mature spermatozoa (Virji and Naz, 1995). Interestingly, this protein is detectable in the peripheral blood of control rats, and induction of germ-cell degeneration by administration of testicular toxicants leads to an increase in these levels (Reader et al, 1991). It is unknown how germ cell-derived proteins enter the bloodstream. They could be resorbed from seminiferous tubule fluid (STF) in the rete testis or epididymis (Maddocks and Sharpe, 1989a) or they could 'leak' via Sertoli cell tight junctions into the interstitium and then enter the bloodstream (Sharpe, 1992).

The objective of the present study was to assess whether seminiferous tubule (ST)-derived proteins could be detected in blood or testicular interstitial fluid (IF) from normal rats, and to assess whether exposure of adult rats to scrotal heating (43°C) for 30 minutes affected the levels of such proteins by inducing germ cell degeneration (Chowdhury and Steinberger, 1964, 1970; Bartlett and Sharpe, 1987; McLaren et al, 1994) and/or by increasing the 'leakiness' of inter-Sertoli cell tight junctions.

Materials and Methods

Animals and Treatments

Young adult Wistar rats ranging in age from 70 to 80 days and bred in the MRC Reproductive Biology Unit were used for all of the studies described below. Animals were housed under conventional conditions (12-hour light:12-hour dark cycle) at a temperature of 21°C and humidity of 35–60%. Food and water were available *ad libitum.*

Scrotal heating of adult rats (43°C for 30 minutes) was performed as described previously (Bartlett and Sharpe, 1987; McLaren et al, 1994). At 24 hours after treatment, the animals were killed, a sample of peripheral venous (PV) blood collected into a heparinized syringe, and the testes removed for collection of interstitial fluid (IF) as described below. Heat treatment was performed on two separate occasions, and each treatment group contained four adult rats. IF and PV samples were also collected from four sham-treated (anesthetized but not heat-exposed) control rats from the same litter or of the same age.

In a single experiment, one adult rat was castrated under anesthesia and administered 1 mg testosterone esters (Sustanon®; Organon Laboratories, Cambridge, UK) subcutaneously (s.c.) every 3 days to maintain approximately normal levels of this steroid in PV blood. Two weeks after the operation, the animal was killed and PV blood collected.

Collection of Samples

Testicular IF was collected overnight (approximately 16 hours) at 4°C, essentially as described by Sharpe and Cooper (1983), although with one important modification. Just prior to isolation of the testes, the efferent ducts were ligated with silk thread to prevent any leakage of rete testis fluid (RTF) into the collecting IF. In one experiment, IF was collected over 30 minutes at 4°C from the testes of four control rats and pooled; the same testes were then maintained overnight at 4°C and a further sample of IF then collected and pooled.

For the collection of plasma samples, animals were anesthetized with ether and heparinized by injection of 125 IU heparin (Leo Laboratories Ltd., Princes Risborough, Bucks, UK) into a femoral vein. The testis was then exposed via a scrotal incision and testicular venous (TV) blood collected into hematocrit tubes by puncturing the major surface testicular vein with a 28-gauge needle at a point just before its division into the mediastinal venous plexus at the anterior pole of the rete (Maddocks and Sharpe, 1989b). Spermatic venous (SV) blood was collected from the spermatic vein towards the top of the pampiniform plexus, on the contralateral testis, by carefully incising one of the veins in the spermatic cord and then allowing spermatic cord blood to drain onto aluminium tinfoil from which it was aspirated. PV blood was then collected from the posterior vena cava and the animal killed by cervical dislocation. All blood samples were centrifuged at 1,000 × g for 30 minutes at 4°C and the resultant plasma stored at −20°C.

Isolation and Culture of Seminiferous Tubules (ST)

ST were isolated and cultured as detailed previously (Sharpe et al, 1992). A total of 10 cm at stages VI–VIII was cultured in 0.4 ml medium containing 4 mM L-glutamine (Sigma, Dorset, UK), 100 IU/ml penicillin and 100 μg/ml streptomycin (Flow Laboratories, Irvine, Scotland, UK), 25 mM HEPES (Gibco, Paisley, Scotland), and 0.1% polyvinyl alcohol (Sigma) in the presence of 60 μCi [^{35}S]methionine (ICN Pharmaceuticals Ltd., Thame, UK) for 22 hours at 32°C under an atmosphere of 5% carbon dioxide and 95% air. After incubation, a protease inhibitor (aprotinin; Sigma) at 0.1% w/v final concentration was added to the seminiferous tubule-conditioned medium (STCM), which was stored at −40°C. Incorporation of [^{35}S]methionine into secreted proteins was determined subsequently by precipitation with trichloroacetic acid (TCA; Sharpe et al, 1992).

Similar methods with minor modifications were used for the preparation of STCM for antibody generation using unstaged tubules (see below). A total of 50 cm of tubules was transferred to the well of a six-well plastic culture plate containing 2 ml culture medium. This consisted of M199, containing Earle's salts and sodium bicarbonate (Flow Laboratories) instead of Eagle's minimal essential medium, and it was supplemented as described above, but excluding the addition of radiolabeled methionine. The tubules were cultured under the same conditions as used for the staged tubules.

Production of Antiserum to STCM

The polyvalent antiserum was generated as follows. STCM was pooled from four rats (total = 32 ml) and lyophilized, reconstituted in 2 ml water, and emulsified with an equal volume of Freund's complete adjuvant. For immunization, two rabbits were each injected with a total of 3.5 mg immunogen at five different intradermal sites. The animals received booster injections at 9

weeks after immunization using 2 mg of immunogen in Freund's incomplete adjuvant, and blood was collected 3 weeks later. The blood was allowed to clot and then centrifuged at 1,000 × *g* for 30 minutes at 4°C; the resultant serum was stored at −40°C. The IgG fraction was prepared from a portion of this antiserum by protein A (Sigma) affinity chromatography using standard methods.

Production of Antiserum to Phosphatidylethanolamine Binding Protein (PEBP)

PEBP is a protein secreted within the testis by round spermatids, which we have isolated and microsequenced previously (Saunders et al, 1995). A peptide antiserum was raised to PEBP using a peptide–carrier protein conjugate (Affiniti Research Products Ltd., Nottingham, UK) to immunize two rabbits. The 20-peptide sequence used was NKSGDHRGKFKVASFRKKYC, corresponding to amino acids 141–159 of the deduced amino acid sequence of monkey epididymal PEBP (Perry et al, 1994). Each rabbit received 0.17 mg peptide in 5 ml of a 1:1 emulsion of phosphate-buffered saline (PBS) and Freund's complete adjuvant, injected at five intradermal sites. Two booster immunizations were administered at 5 and 10 weeks after the primary immunization, using 0.17 mg peptide in 5 ml of a 1:1 mixture of PBS and Freund's incomplete adjuvant. Blood samples were then collected 2 weeks after the booster injections, the blood was allowed to clot and centrifuged at 1,000 × *g* for 30 minutes at 4°C, and the resultant serum was stored at −40°C.

Isolation of Leydig Cells

Because putative germ cell-derived proteins were detected in testicular IF, the possibility that homologous proteins might originate from Leydig cells was investigated. Leydig cells were isolated from the testes of rats (aged 80–120 days) by collagenase digestion, followed by fractionation on discontinuous Percoll gradients (Simpson et al, 1987).

A total of 100,000 cells, of which 76–88% were histochemically identifiable as Leydig cells, based on staining for 3β-hydroxysteroid dehydrogenase, was incubated in 300 μl Eagle's minimal essential medium without methionine (Flow Laboratories). This was supplemented with 4 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 25 mM HEPES, 0.1% polyvinyl alcohol as a protein substitute, and 60 μCi [^{35}S]-methionine (ICN Pharmaceuticals Ltd.); the latter was thus present at a final concentration of 0.75 μM methionine per well. Cells were incubated for 20 hours at 32°C, and the medium was aspirated and stored at −20°C. Incorporation of [^{35}S]methionine into secreted proteins was determined subsequently by precipitation with TCA (Sharpe et al, 1992).

One-dimensional (1-D) Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoresis was performed using a Protean II electrophoresis system and a model 3000xi power supply (Bio-Rad, Hemel Hempstead, UK). The apparatus was cooled to 10°C using a chiller/heater circulator unit (Betta-Tech Controls, Newport Pagnell, Bucks, UK). Sample separation was performed using 7–15% w/v gradient acrylamide gels. The wells were loaded with specific amounts of protein depending on the sample (STCM 2 μg; IF and all plasma samples, 100 μg). Protein concentration was estimated using a protein assay reagent (Bio-Rad) based on dye binding (Bradford, 1976) using a bovine serum albumin (BSA) standard (Bio-Rad). Samples were mixed with an equal volume of SDS sample buffer containing 1.5% w/v Tris, 4% w/v SDS, 2% w/v dithiothrietol (DTT) (all from Sigma), and 0.05% w/v bromophenol blue (Bio-Rad), boiled for 5 minutes, centrifuged at 1,000 × *g* for 3 minutes, and then loaded into the wells of the stacking gel. The electrolyte buffer contained 0.3% w/v Tris base, 1.44% w/v glycine, and 0.1% w/v SDS (all from Sigma). After electrophoresis, the gel was electroblotted for Western blot analysis (see below).

Two-dimensional (2-D) SDS-PAGE

The materials and methods used for 2-D SDS-PAGE were as detailed previously (Sharpe et al, 1992). Second-dimension separations of the radiolabeled Leydig cell- or ST-conditioned medium utilized 10% w/v acrylamide gels; samples to be Western blotted were separated on 7–15% w/v acrylamide gradient gels. After electrophoresis, gels were either electroblotted or fixed and silver stained using a kit (Bio-Rad) according to the manufacturer's instructions. Those gels used to resolve the radiolabeled ST- and Leydig cell-secreted proteins were soaked in Amplify (Amersham International) after being silver stained and dried for about 2 hours at 62°C on a model 543 gel drier (Bio-Rad). Dried gels were exposed to x-ray film (X-OMAT ARS; Kodak Scientific Imaging Systems Limited, Cambridge, UK) at −80°C for 21 days.

Western Blotting

Samples of IF and PV were resolved on both 1-D and 2-D gels and subjected to Western blot analysis using the IgG fraction of the polyvalent antiserum raised in rabbits against STCM to establish whether ST-secreted proteins were detectable. Following electrophoresis, gels were electroblotted by semi-dry transfer onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Watford, UK), using a Novablot apparatus (LKB Pharmacia, Uppsala, Sweden). The transfer buffer contained 0.06% w/v Tris base, 0.288% w/v glycine, 0.02% w/v SDS (all from Sigma), and 20% w/v methanol. Membranes were blocked for 2 hours at room temperature with gentle shaking in TBS-Tween (Tris-buffered saline, pH 7.4, containing 50 mM Tris-HCl, 150 mM NaCl, and 0.05% w/v Tween 20), to which was added 5% w/v normal sheep serum (SAPU Laboratories, Carluke, Scotland). After blocking, the membranes were incubated overnight at 4°C with either primary antibody diluted 1:500 in blocking buffer or with normal rabbit serum (Dako, High Wycombe, UK) diluted to an equivalent protein concentration as the antiserum for control purposes. Unbound antibodies were removed by three washes for 10 minutes in TBS-Tween, followed by a 45-minute incubation with secondary antibody (sheep anti-rabbit IgG; Serotec, Oxford, UK) diluted 1:500 in TBS. This and all subsequent steps were performed at room temperature with gentle shaking. After a further three washes for 10 minutes in TBS-Tween, membranes were incubated for 45 minutes in rabbit alkaline phosphatase anti-alkaline phosphatase conjugate

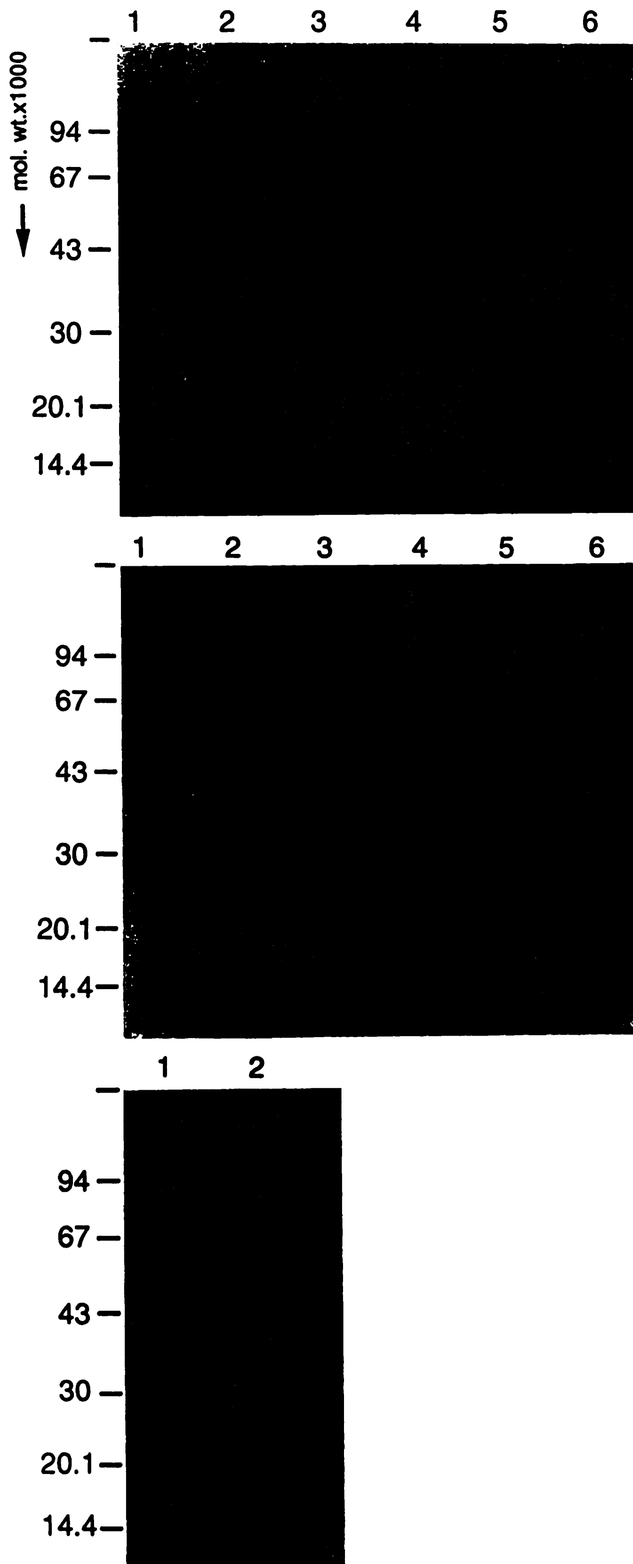

FIG. 1. A representative Western blot showing the detection of seminiferous tubule (ST)-secreted proteins in ST-conditioned medium (STCM), testicular interstitial fluid (IF), and samples of testicular (TV), spermatic (SV), and peripheral (PV) venous plasma from control adult rats. The samples were resolved using one-dimensional (1-D) sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on a 7–15% w/v acrylamide gel. After electrophoresis, the proteins were electroblotted onto a membrane and detected using the IgG fraction of an antiserum raised against STCM **(a)** or control rabbit serum **(b)** as the primary antibody. **Lane 1**, STCM (2 μg protein); **lane 2**, IF (100 μg); **lane 3**, SV (100 μg); **lane 4**, TV (100 μg); **lane 5**, PV (100 μg); and **lane 6**, PV (100 μg) from a castrated rat supplemented with testosterone. In panel **(c)**, the gel was loaded with IF (100 μg) collected over 30 minutes **(lane 1)** and also over 16 hours **(lane 2)**. Three proteins detected by the antiserum (A, B, and C: denoted by arrows) present in both STCM and IF are indicated.

(Serotec) diluted 1:2,000 in TBS. Membranes were then washed twice for 10 minutes in TBS-Tween, once for 10 minutes in TBS alone, and then equilibrated for 2 minutes in buffer containing 100 mM Tris, 100 mM NaCl, and 50 mM $MgCl_2$. Blots were developed immediately in 25 ml equilibration buffer to which was added 0.3 mg/ml nitro blue tetrazolium (NBT), 0.175 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate, and 0.24 mg/ml levamisole (all from Sigma). Developing was carried out in the dark until staining was optimal.

All 1-D Western blots, which utilized the antiserum to STCM, were repeated at least twice using individual samples of IF and PV from a minimum of eight different control and six different heat-treated rats. IF and PV samples from at least three different control and three different heat-treated animals were subjected to 2-D Western blotting. Samples of STCM and IF were also Western blotted, using the antiserum raised against a PEBP peptide, to confirm the identity of one of the proteins recognized by the antiserum to STCM. This antiserum was used at a 1:500 dilution with the same protocol as described above for the antiserum to STCM.

Results

Detection of ST-secreted Proteins Using the Antiserum to STCM

Using the antiserum to STCM, Western blot analysis of STCM (the immunogen) prepared from control adult rats revealed that, as expected, the antiserum recognized several specific proteins (Fig. 1a, lane 1) that were not detected when blotted using normal rabbit serum (Fig. 1b, lane 1). Three proteins that were present in STCM were also detected by Western blotting in every sample of IF from control rats ($n = 8$ in total) that was analyzed; their molecular masses were estimated to be 24 (A), 16 (B), and 14 kDa (C), respectively (Fig. 1a, lane 2). These proteins were equally evident in Western blots of IF that had been collected over 30 minutes (Fig. 1c, lane 1) or over 16 hours at 4°C (Fig. 1c, lane 2), indicating that their presence in the latter was not because of their artifactual release during the overnight collection period. Western blot analysis of plasma, whether of TV, SV, or PV, failed to detect any proteins in the plasma samples that were not also evident with the control rabbit serum (Fig. 1a and b, lanes 3–5). Similar results were obtained using PV collected from a rat 2 weeks after castration, in which

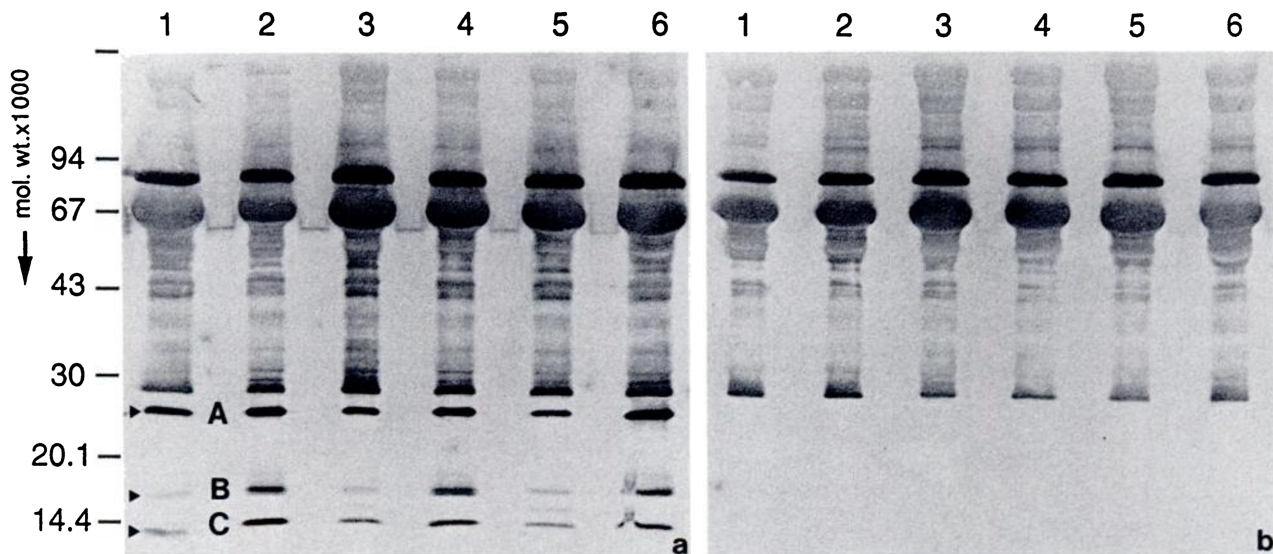

FIG. 2. Representative Western blots showing the detection of seminiferous tubule (ST)-secreted proteins in samples of testicular interstitial fluid (a) and peripheral venous plasma (b) from control adult rats and from animals in which spermatogenesis had been impaired by heating the scrotum (43°C for 30 minutes) 24 hours earlier. Samples were resolved on 7–15% w/v acrylamide gels by 1-D SDS-PAGE, then electroblotted onto a membrane and probed with an antiserum raised against ST-secreted proteins. In panel (a), lanes 1, 3, and 5 were loaded with IF (100 μg protein) from three different control rats; lanes 2, 4, and 6 were loaded with IF (100 μg) from three different heat-treated rats. In panel (b), corresponding samples of PV plasma were blotted (lanes 1, 3, and 5: three control rats; lanes 2, 4, and 6: three heat-treated rats). Three proteins (A, B, and C) that showed changes in abundance in IF from control and heat-treated animals are indicated by arrows.

testosterone levels had been restored by injection (Fig. 1a and b, lane 6). Although several proteins greater than 27 kDa were detected in IF and the plasma samples, it was not possible to analyze these effectively because normal rabbit serum also bound nonspecifically to proteins in this molecular weight range.

Effects of Testicular Heat Treatment on the Detection of ST-secreted Proteins in IF and PV by Western Blot Analysis

Western blot analysis was used to assess whether the abundance of the proteins A, B, and C detected in control IF changed, or if any new proteins could be detected, after damage to spermatogenesis was induced by scrotal heating 24 hours earlier (Fig. 2a, lanes 2, 4, and 6). All three proteins increased in abundance in each of three IF samples from individual heat-treated animals compared with IF samples from three individual control rats (Fig. 2a, lanes 1, 3, and 5), as judged by the greater NBT staining intensity; this increase was less marked for protein A than for proteins B and C. An additional faint band, with a molecular mass of approximately 18 kDa, was detected in IF samples from heat-treated animals, although this band is difficult to visualize on photographs of the Western blots. These changes were observed in all samples of IF from heat-treated rats ($n = 6$) that were analyzed.

PV samples from control and heat-treated rats were also subjected to Western analysis, using the antiserum to STCM, to ascertain whether disruption of spermatogenesis might lead to the appearance of previously undetectable ST-secreted proteins in the bloodstream. However, this analysis failed to detect any proteins originating from the ST in any of the samples (Fig. 2b).

Investigation of the Protein Changes Caused by Heat Treatment Using 2-D Western Blots

Western blot analysis using 2-D SDS-PAGE was undertaken to provide additional information on possible identities of the ST-derived proteins detected in IF by comparison with the 2-D profile of STCM, for which a number of the proteins have been characterized and their cellular origin established (Sharpe et al, 1994; McKinnell and Sharpe, 1995). This analysis confirmed that heat-induced testicular damage led to an increased leakage of proteins from the seminiferous tubules (Fig. 3, compare panel b with a). Protein A (24 kDa) appeared as four distinct charge isomers in both STCM and IF, and these proteins appeared to be increased in IF samples from heat-treated rats (Fig. 3b). In the latter, a further three proteins were detected that resolved with the same isoelectric point (pI) as protein A but with a slightly lower molecular weight, raising the possibility that they are related to protein A. Protein B (16 kDa) could not be detected in IF from control or heat-treated animals on the 2-D Western blots, in contrast to the results on the 1-D blots. This probably means that the pI of this protein is not within the range (4.8–8.0) that was resolved on the 2-D gel. On the 1-D blots, protein C (14 kDa) showed an increase in abundance

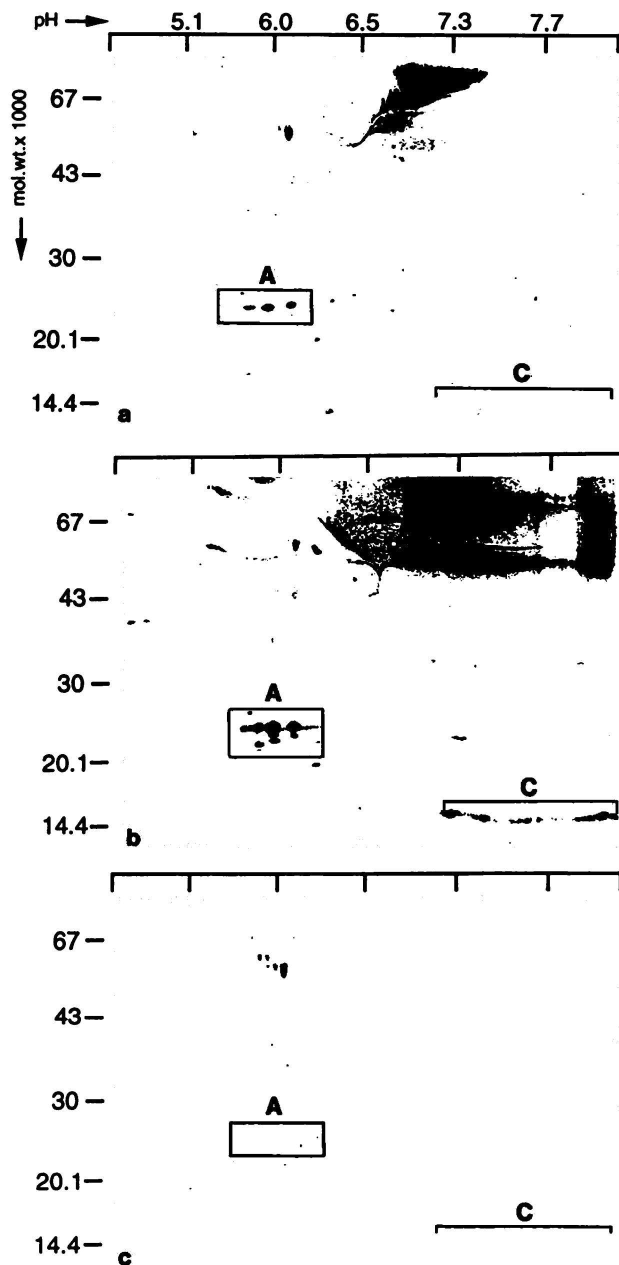

FIG. 3. Representative Western blots of testicular IF (500 μg protein) resolved by two-dimensional (2-D) SDS-PAGE on 7–15% w/v acrylamide gels, electroblotted onto a membrane and probed with an antiserum raised against STCM (panels a and b) or with normal rabbit serum (panel c). In panels (a) and (c), the gel was loaded with IF from a control rat, whereas IF from a heat-treated rat was used in panel (b). Proteins A and C, identified by 1-D Western blot analysis, are labeled where possible.

in IF from heat-treated rats compared with control rats; on the 2-D blots it appeared as a broad unresolved smear and stained very intensely in both STCM from normal rats (data not shown) and in IF from heat-treated animals (Fig. 3b). Several other less abundant proteins (approximately 20) were detected in IF samples after heat-treatment that were not present in the control samples, although none of these have been reproduced in the photograph of the blot. PV samples from control and heat-treated rats were also subjected to 2-D Western blot analysis, but this approach failed to detect any of the immunoactive proteins found in IF (results not shown).

The position of proteins A and C is indicated on each of the Western blots (Fig. 3). Based on their molecular weights and pI values, proteins A and C were identified tentatively as PEBP (Saunders et al, 1995) and ARP-2 (Sharpe et al, 1992; McKinnell and Sharpe, 1995), respectively, both of which derive specifically from round and elongating spermatids. The sequence and nature of ARP-2 is unknown. Confirmation that protein A was PEBP was obtained by Western blotting using an antiserum generated against a PEBP peptide. A protein band of 24 kDa, resolving on 2-D blots as several charge isomers, was detected in STCM using this antiserum (Fig. 4). This corresponds to the molecular weight and pI of PEBP (Saunders et al, 1995). A few fainter-staining bands of a higher molecular weight were also detected that may be nonspecific, because comparable staining was evident in an equivalent blot incubated with normal rabbit serum (data not shown). The 24-kDa band was detected with the PEBP antiserum in all IF samples and was found to be increased after heat treatment (Fig. 4).

Origin of ST-derived Proteins Detected in IF

Although our previous studies have shown that PEBP (protein A) and ARP-2 (protein C) originate from germ cells, the possibility that these two proteins might derive from Leydig cells or a peripheral source was considered. Both of these proteins were secreted by ST when they were cultured in the presence of [^{35}S]methionine (Fig. 5a), but no corresponding radiolabeled proteins were detected in medium from cultured Leydig cells (Fig. 5b), indicating that their presence in IF is unlikely to be due to secretion by Leydig cells. Moreover, the STCM antiserum, which recognizes on 2-D Western blots nearly 100 proteins (data not shown) secreted *in vitro* by isolated ST, failed to detect any corresponding proteins in TV, SV, or PV blood samples; the proteins A and C in IF are therefore unlikely to derive from the bloodstream.

Discussion

The primary purpose of this investigation was to assess whether proteins deriving from the seminiferous epithelium can gain access to the bloodstream and thus provide a potential means of monitoring the process of spermatogenesis. An antiserum was raised against STCM (ST-secreted proteins) and, in conjunction with Western blot analysis, was used to screen IF and blood samples from control adult rats and rats in which damage to spermato-

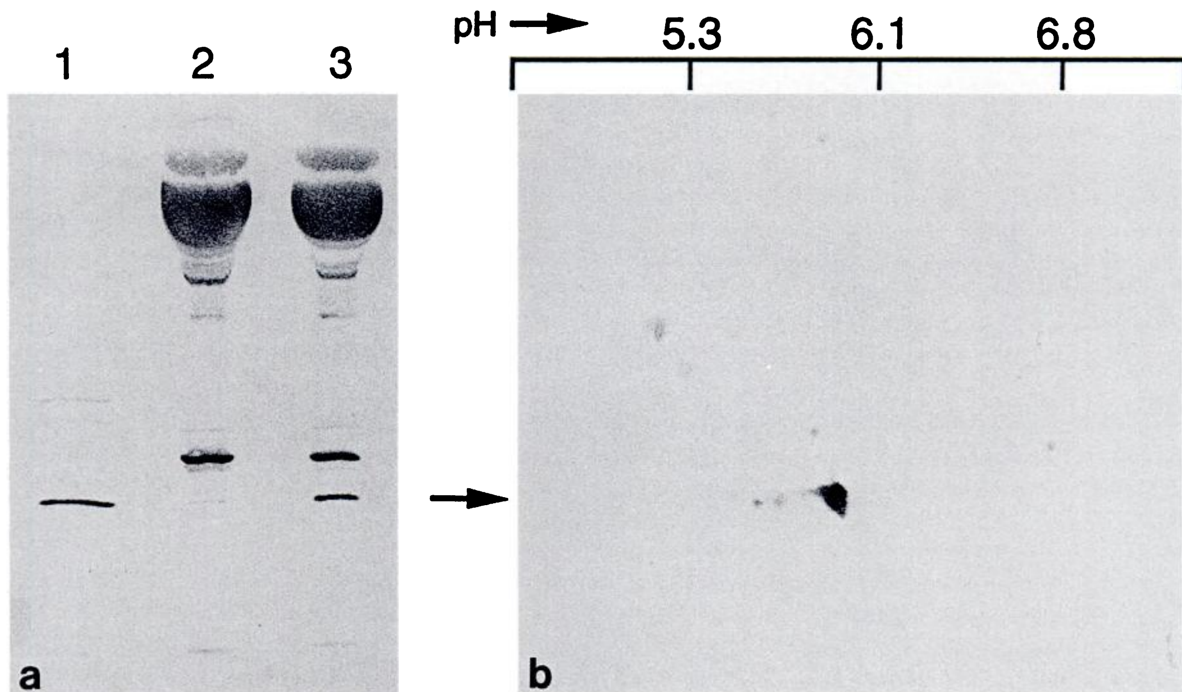

FIG. 4. Identification of the 24-kDa protein, present in both STCM and IF, as PEBP, by Western blot analysis using an antiserum generated against a PEBP peptide. In panel **(a)**, STCM and IF from control and heat-treated animals was resolved on a 7–15% w/v acrylamide gel by 1-D SDS-PAGE and then blotted. **Lane 1**: STCM (10 μg protein); **lane 2**: IF (100 μg) from a control rat; **lane 3**: IF (100 μg) from a heat-treated rat. In panel **(b)**, STCM (30 μg) was resolved by 2-D SDS-PAGE on a 15% acrylamide gel. Arrows indicate the position of PEBP on both Western blots.

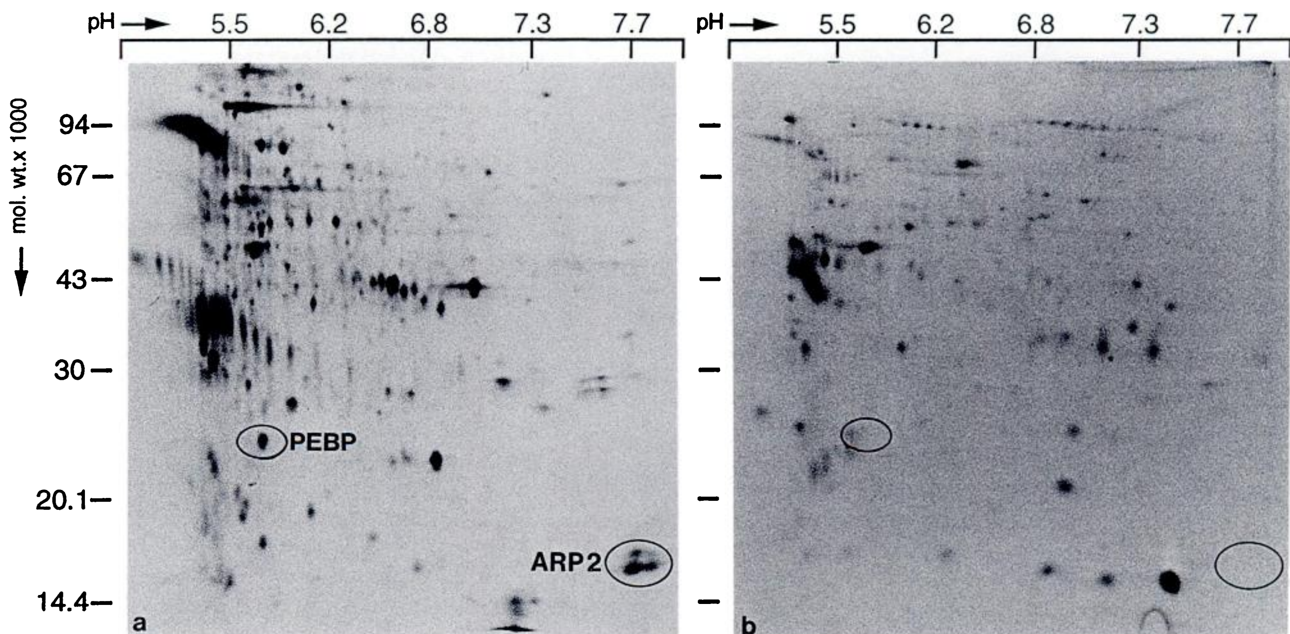

FIG. 5. Representative fluorographs of ^{35}S-labeled proteins secreted *in vitro* by ST isolated at stages VI–VIII of the spermatogenic cycle **(a)** or by Percoll-purified Leydig cells **(b)** from control adult rats. Radiolabeled secreted proteins (300 × 10^3 cpm) were resolved by 2-D SDS-PAGE on 10% w/v acrylamide gels. The position of PEBP and ARP-2 is marked on the ST autoradiograph and their absence indicated on the Leydig cell autoradiograph.

genesis had been induced by heat treatment 24 hours earlier. Using this antiserum, three proteins were detected in testicular IF from control rats. Heat treatment increased the abundance of these proteins and induced the appearance of several other proteins that were recognised by the STCM antiserum. Two proteins present in IF from control rats and which were increased in abundance by testicular damage were identified as PEBP and ARP-2, both of which are known to be major secretory products of round spermatids (Sharpe et al, 1992, 1994; Saunders et al, 1995; McKinnell and Sharpe, 1995). These results suggest that germ cell secretory products can gain access to the interstitium under both normal physiological conditions and more easily after induction of damage to spermatogenesis. This may mean that if suitable methodology can be devised, germ cell proteins could also be detectable in blood, thus providing a noninvasive way of monitoring spermatogenesis. This possibility was investigated in a preliminary way in the present studies using Western blotting, but no proteins recognised specifically by the STCM antiserum in IF were detectable in blood samples. It seems likely that this negative result is the consequence of the insensitivity of the present techniques, which were limited technically by the protein-resolving capacity of the acrylamide gels (100 and 500 μg total protein for 1-D and 2-D gels, respectively). Because both IF and plasma have a similar protein concentration (approximately 50 μg protein per μl), only 2–10 μl of sample can be analyzed by this method. Development and application of specific immunoassays for individual ST-derived proteins (e.g., PEBP) should overcome this drawback and enable unequivocal assessment of whether such proteins are present in PV blood under normal or experimental conditions (e.g., after heat treatment). In view of our findings, PEBP and ARP-2 are obvious candidates for such investigations.

Detection of germ cell-derived proteins in IF with the STCM antiserum could be due to contamination of the IF by rete testis fluid or the consequence of mechanical damage to the ST during the IF collection procedure. The former possibility was ruled out by ligating the efferent ducts prior to testicular isolation and collection of IF. We cannot completely rule out the possibility of mechanical damage during the IF collection procedure, but three pieces of evidence suggest that this is unlikely to explain our findings. First, IF collected over periods of either 30 minutes or 16 hours at 4°C exhibited similar protein profiles by Western analysis, indicating that lysis of cells or artifactual disruption of inter-Sertoli cell junctions during the longer of these collection periods did not explain the presence of germ cell-derived proteins in IF. Second, nonspecific mechanical damage would be expected to result in the release of a wider (and perhaps more variable) range of ST-derived proteins, detectable by the STCM antiserum, rather than the three specific proteins that were detected in every sample of IF analyzed. Of significance in this respect is that all of the ST-derived proteins detected by Western blotting in IF were below 25 kDa in molecular weight, whereas the STCM antiserum also recognized many proteins of much higher molecular weight. In contrast, 'natural leakage' of inter-Sertoli cell junctions would be expected to preferentially affect low molecular weight proteins, and this is what was observed. Third, mechanical trauma would fail to explain why these specific proteins were increased in abundance in IF collected from heat-treated animals, whereas this treatment is known to increase leakage/secretion of Sertoli cell-derived proteins into IF (see Sharpe, 1992 for references).

Another explanation that we have considered for our findings is that homologues of the ST-derived proteins detected in IF are secreted by Leydig cells or by a peripheral source. Again, several pieces of evidence indicate that this is unlikely. First, no proteins corresponding in molecular weight and pI to PEBP and ARP-2 were secreted by isolated Leydig cells. Second, immunostaining of the mouse testis with an antiserum to PEBP (Vierula et al, 1992) and analysis of PEBP mRNA by *in situ* hybridization on adult rat testis (Saunders et al, 1995) showed no reaction with peritubular cells or cells of the interstitium. Therefore, the available data indicate that this protein is unlikely to originate from a testicular source other than germ cells. Because none of the proteins detected in IF by the STCM antiserum were detectable in any of the blood samples, a peripheral source of these proteins can presumably also be ruled out. This thinking is reinforced by the fact that PEBP seems to be a nonsecreted cytosolic protein outside of the male reproductive tract (Jones and Brown, 1987; Jones and Hall, 1991; Saunders et al, 1995).

The presence of germ cell proteins in the IF compartment of the testis in control adult rats is a surprising observation. The inter-Sertoli cell tight junctions act as a selective permeability barrier between IF and STF (Pelletier and Byers, 1992; Setchell et al, 1994), and the transport of molecules from the IF compartment into STF is little affected by heat treatment (Main and Waites, 1977). However, the leakiness of the barrier in the opposite direction has been poorly studied for proteins other than those deriving from Sertoli cells (Sharpe, 1992). Our results suggest that germ cell secretions can gain access to IF, presumably by leakage, although it is conceivable that they might be transported actively across the Sertoli cells. This observation has interesting implications concerning the possible function of these germ cell proteins in the interstitium. It is well established that depletion of germ cells from ST can result in marked changes in Leydig cell structure and function (Sharpe, 1993), effects that are thought to be mediated by effects on the Sertoli cells. However, the present observations suggest that it is per-

haps possible for germ cell secretions to directly influence Leydig cells.

This study has provided preliminary evidence that when spermatogenesis is disrupted by heat exposure, increased numbers and amounts of proteins deriving from the seminiferous tubules can be found in IF. Such proteins therefore have the potential to be diagnostic markers of spermatogenic impairment. However, the present results also demonstrate clearly that more sensitive methods will have to be developed if noninvasive monitoring of spermatogenesis using such an approach is to become a realistic probability. This will involve targeting of specific proteins for isolation and sequencing (McKinnell and Sharpe, 1995) and then the generation of specific antibodies that can be used to establish assays for the sensitive detection of such proteins in blood. The present results have identified two possible candidates for such an approach, and because a homologue of one of these proteins may be secreted by human ST (ARP-2; McKinnell et al, 1995), this approach may have clinical applications. The present observation that the large number of proteins secreted by isolated ST *in vitro,* and that are recognized by our polyvalent antiserum to STCM, do not appear to have homologues of major abundance in peripheral blood is an encouraging finding in terms of the potential specificity of any assays for ST-derived proteins that might be developed.

Acknowledgments

We are grateful to Dennis Doogan for his skilled assistance. K.J.T. was supported by a Partnership award between the MRC and Zeneca plc, S.J.Q. was supported by ICI Agrochemicals Ltd., and T.T.McL. was in receipt of a Fellowship from the *Journal of Reproduction and Fertility Ltd.*

References

Bartlett JMS, Sharpe RM. Effect of local heating of the rat testis on the levels in interstitial fluid of a putative paracrine regulator of the Leydig cells and its relationship to changes in Sertoli cell secretory function. *J Reprod Fertil* 1987;80:279–287.

Bradford MM. A rapid and sensitive method for the quantitative measurement of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248–254.

Cheng CY, Bardin CW. Rat testicular testibumin is a protein responsive to follicle stimulating hormone and testosterone that shares immunodeterminants with albumin. *Biochemistry* 1986;25:5276–5288.

Cheng CY, Frick J, Gunsalus GL, Musto NA, Bardin CW. Human testicular androgen-binding protein shares immunodeterminants with serum testosterone-estradiol-binding globulin. *Endocrinology* 1984; 114:1395–1401.

Cheng CY, Grima J, Lee WM, Bardin CW. The distribution of rat testibumin in the male reproductive tract. *Biol Reprod* 1987;37:875–885.

Chowdhury AK, Steinberger E. A quantitative study of the effect of heat on germinal epithelium of rat testes. *Am J Anat* 1964;15:509–524.

Chowdhury AK, Steinberger E. Early changes in the germinal epithelium of rat testes following exposure to heat. *J Reprod Fertil* 1970;22:205–212.

Jenne DE, Tschopp J. Molecular structure and functional characterization of a human complement cytolysis inhibitor found in blood and seminal plasma: identity to sulphated glycoprotein 2, a constituent of rat testis fluid. *Proc Natl Acad Sci USA* 1989;86:7123–7127.

Jones R, Brown CR. Identification and characterization of the 2D6 and Mr 23000 antigens on the plasma membrane of rat spermatozoa. *Biochem J* 1987;241:353–360.

Jones R, Hall L. A 23 kDa protein from rat sperm plasma membranes shows sequence similarity and phospholipid binding properties to a bovine brain cytosolic protein. *Biochim Biophys Acta* 1991;1080:78–82.

de Kretser DM, McLachlan RI, Robertson DM, Burger HG. Serum inhibin levels in normal men and men with testicular disorders. *J Endocrinol* 1989;120:517–523.

de Kretser DM, Robertson DM. The isolation and physiology of inhibin and related proteins. *Biol Reprod* 1989;40:33–47.

Maddocks S, Sharpe RM. The route of secretion of inhibin from the rat testis. *J Endocrinol* 1989a;120:R5–R8.

Maddocks S, Sharpe RM. Dynamics of testosterone secretion by the rat testis: implications for measurement of intratesticular levels of testosterone. *J Endocrinol* 1989b;122:323–329.

Main SJ, Waites GMH. The blood–testis barrier and temperature damage to the testis of the rat. *J Reprod Fertil* 1977;51:439–450.

McKinnell C, Brackenbury ET, Qureshi SJ, Hargreave TB, Sharpe RM. Comparative analysis of proteins secreted *in vitro* by isolated seminiferous tubules from man and the rat. *Int J Androl* 1995;18:103–111.

McKinnell C, Sharpe RM. Testosterone and spermatogenesis: evidence that androgens regulate cellular secretory mechanisms in stage VI–VIII seminiferous tubules from the adult rat. *J Androl* 1995;16:499–509.

McLachlan RI, Robertson DM, de Kretser DM, Burger HG. Advances in the physiology of inhibin and inhibin-related peptides. *Clin Endocrinol* 1988;29:77–114.

McLaren TT, Foster PMD, Sharpe RM. Identification of stage-specific changes in protein secretion by isolated seminiferous tubules from rats following exposure to short-term local testicular heating. *J Reprod Fertil* 1994;102:293–300.

Pelletier RM, Byers SW. The blood–testis barrier and Sertoli cell junctions: structural considerations. *Microsc Res Tech* 1992;20:3–33.

Perry ACF, Hall L, Bell AE, Jones R. Sequence analysis of a mammalian phospholipid-binding protein from testis and epididymis and its distribution between spermatozoa and extracellular secretions. *Biochem J* 1994;301:235–242.

Reader SCJ, Shingles C, Stonard MD. Acute testicular toxicity of 1,3-dinitrobenzene and ethylene glycol monomethyl ether in the rat: evaluation of biochemical effect markers and hormonal responses. *Fundam Appl Toxicol* 1991;16:61–70.

Saunders PTK, McKinnell C, Millar MR, Gaughan J, Turner KJ, Jegou B, Syed V, Sharpe RM. Phosphatidylethanolamine binding protein is an abundant secretory product of haploid testicular germ cells in the rat. *Mol Cell Endocrinol* 1995;107:221–230.

Setchell BP, Maddocks S, Brooks DE. Anatomy, vasculature, innervation, and fluids of the male reproductive tract. In: Knobil E, Neill JD, eds. *The Physiology of Reproduction.* 2nd ed. New York: Raven Press; 1994:1063–1175.

Sharpe RM. Monitoring of spermatogenesis in man—measurement of Sertoli cell- or germ cell-secreted proteins in semen or blood. *Int J Androl* 1992;15:201–210.

Sharpe RM. Experimental evidence for Sertoli–germ cell and Sertoli–Leydig cell interactions. In: Russell LD, Griswold MD, eds. *The Sertoli cell.* Clearwater, Florida: Cache River Press; 1993:391–418.

Sharpe RM, Cooper I. Testicular interstitial fluid as a monitor for changes in the intratesticular environment in the rat. *J Reprod Fertil* 1983; 69:125–135.

Sharpe RM, Maddocks S, Millar M, Saunders PTK, Kerr JB, McKinnell C. Testosterone and spermatogenesis: identification of stage-dependent, androgen-regulated proteins secreted by adult rat seminiferous tubules. *J Androl* 1992;13:172–184.

Sharpe RM, McKinnell C, McLaren TT, Millar MR, West AP, Maguire SM, Gaughan J, Syed V, Jegou B, Kerr JB, Saunders PTK. Interactions between androgens, Sertoli cells and germ cells in control of spermatogenesis. In: Verhoeven G, Habenicht UF, eds. *Molecular and Cellular Endocrinology of the Testis: Ernst Schering Research Foundation Workshop.* Suppl 1. Berlin: Springer-Verlag; 1994:115–142.

Simpson BJB, Wu FCW, Sharpe RM. Isolation of human Leydig cells which are highly responsive to human chorionic gonadotrophin. *J Clin Endocrinol Metab* 1987;65:415–422.

Vierula ME, Araki Y, Rankin TL, Tulsiani DP, Orgebin-Crist M-C. Immunolocalization of a 25-kilodalton protein in mouse testis and epididymis. *Biol Reprod* 1992;47:844–856.

Virji N, Naz RK. The role of lactate dehydrogenase-C_4 in testicular function and infertility. *Int J Androl* 1995;18:1–7.