Experimental studies on the passage of specific IgG to the lumen of the rabbit epididymis†

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Summary. The extent to which specific IgG can reach the lumen of the rabbit cauda epididymidis was investigated by comparison of the concentration in serum and fluid of the cauda epididymidis of a specific IgG raised against dinitrophenylated bovine gamma globulin (DNP-BGG). This specific IgG reached the epididymal lumen although in much lower concentration than the levels in serum. The IgG was measured by a specific sensitive radioimmunoassay and in 13 normal males there was a mean molar ratio of 4.0×10^{-3} (range: 2.5 – 11.0×10^{-3}) between the epididymal lumen and blood; the mean ratio between cerebrospinal fluid and blood was 1.7×10^{-3} (4 males). Calculations, based on the absolute concentration of anti-DNP IgG in epididymal fluid in relation to total number of spermatozoa and estimated fluid volume in the cauda epididymidis, indicated approximately 40 000 molecules anti-DNP-BGG IgG per spermatozoon. This ratio was not affected 6 days after castration or 3–4 months after vasectomy, but it was about 10 times higher than that of the controls in the cryptic epididymis subjected for 6 days to body temperature.

Introduction

Much has been learned in recent years about the functions of the epididymis in the maturation and storage of spermatozoa. However, little is known of the extent to which biological agents may pass from the blood to the epididymis. Neither is it clear how this may vary according to species or the site in the epididymis being explored. Passage of foreign components from the systemic circulation to the lumen of the rat caput epididymidis appears to be determined to a considerable extent by the molecular weight of the entity in question; larger molecules such as inulin and bovine serum albumin do not reach more than about 5% of the concentration in the blood, whereas tritiated water equilibrates much more freely with the epididymal compartment (Hinton & Howards, 1981). The same appears to be true in principle for the rat cauda epididymidis when studied by luminal perfusion techniques, this region seeming relatively impermeable to larger molecules (Cooper & Waites, 1979).

Whether or not such principles apply to immunoglobulins (Igs) evoked by systemic immunization is not known. The luminal border of the epididymal epithelium is characterized by impressive junctional complexes (Friend & Gilula, 1972; Suzuki & Nagano, 1978), and while occasional lymphocytes can be detected within the epithelium (Dym & Romrell, 1975), there is no significant population of antibody-secreting cells in the lumen of the normal duct. However, Igs may reach the lumen of the gut by transudation (Tomasi, 1976) and there is evidence that

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some proteins reach the interstitium of the epididymis by this means (Kormano, 1968; Johnson, 1972). Nonetheless, too little is known to be able to predict the Ig concentration ratio between the blood and the epididymal lumen, particularly since local Ig production could affect the final concentration appearing in the epididymis. Mancini, Vilar, Alvarez & Sequier (1965) concluded that such proteins may enter the epididymis from the testis, but others using immunofluorescent methods found no Ig-positive cells or precipitate in the lumen (Johnson, 1972; Alexander & Fulgham, 1978).

It is now possible to recover uncontaminated fluids from the distal epididymis, and to measure Ig in very small fluid volumes by quantitative radioimmunoassay of high sensitivity and specificity. We have used these methods to investigate (a) the extent to which IgG raised against dinitrophenylated bovine gamma globulin passes to the epididymal lumen, (b) the specific IgG concentration ratio established between this compartment and blood, and (c) the effects of an elevated temperature, of androgen withdrawal, and of vasectomy on that ratio.

Materials and Methods

Reagents. Dinitrophenylated-bovine gamma globulin (DNP-BGG) and -ovalbumin were synthesized by standard methods using dinitrofluorobenzene or dinitrobenzenesulphonic acid (Eastman Chemicals). Ovalbumin and bovine gamma globulin were obtained commercially. Reaction conditions were those of Porter & Sanger (1948). Unreacted DNP groups were removed by passage over Sephadex G-25. Rough estimates of DNP substitution rates were calculated by comparison of absorption at 360 and 280 nm. For the purpose of immunization, protein concentrations were taken as a function of the absorbance at 280 nm, assuming a non-substituted protein. This method was confirmed using known amounts of substituted and non-substituted proteins. An emulsion for immunization was made in a Virtis Laboratory Emulsifier by adding roughly equal volumes of a solution of 2 mg DNP-BGG/ml and complete Freund's Adjuvant.

Affinity-purified anti-DNP was prepared from sera of rabbits which had been repeatedly boosted over 3–4 months and whose sera showed, by Ouchterlony analysis against DNP-ovalbumin, strong reactions that would be inhibited by DNP-glycine. The affinity column was prepared by linking DNP-ovalbumin to sepharose beads using cyanogen bromide as a cross-linking reagent under alkaline conditions (see Fuchs & Sela, 1978). The DNP-ovalbumin-sepharose was poured into a glass column and washed with buffer to non-detectable absorption at 280 nm. A 50% ammonium sulphate precipitate of serum was resuspended and dialysed in buffer and passed over the column. After the non-affinity proteins were eluted, the column was again washed with buffer until absorption at 280 nm was <0.010 . The anti-DNP Ig was then eluted using 0.2 M-glycine-HCl at pH 2.5 with the collected fractions being 'quenched' to pH 5.5 by 10 volumes of buffer in the fractionation tubes. Ouchterlony analysis showed that this material reacted with DNP-ovalbumin and goat anti-rabbit Ig. Polyacrylamide slab gel electrophoresis, run according to Laemmli (1970) (10% with SDS: reduced by 2-mercaptoethanol and heat denatured), revealed that $>90\%$ of the material was made up of heavy and light chains. This material, quantified by absorption at 280 nm, was used as 'standard Ig anti-DNP'.

Solid-phase supports for the radioimmunoassay (RIA) were flexible 96-well microtitre plates (Linbro).

Staphylococcus protein A (Pharmacia) was iodinated by the chloramine-T or peroxidase method under standard conditions. Separation from unreacted ^{125}I was carried out by Sephadex G-25 elution and the radioactivity was shown to be $>95\%$ protein-bound by dialysis and acid precipitation. Typical specific radioactivities were greater than 10^{17} c.p.m./mol.

Immunization procedures. Ejaculates were collected from adult males by means of an

artificial vagina; rabbits with an ejaculate of normal appearance were selected for use. The immunizing reagent, DNP-BGG, at 1 mg/ml concentration, was injected into each of the front footpads (approximately 0.5 ml per foot). After 4–6 weeks, blood was taken to monitor circulating levels of specific anti-DNP and a booster injection of 0.1 mg DNP-BGG alone was given intravenously. This sequence of bleeding and boosting was carried out approximately every 2 weeks until high serum titres of anti-DNP were reached. When high titres were evident, the males were then used for collection of epididymal fluids as described below.

Radioimmunoassay. The RIA was of the solid-phase type. DNP-ovalbumin at 1 mg/ml was used to coat the wells of a linbro microtitre plate by passive absorption over 24 h at 4°C. The DNP-ovalbumin was removed and all wells (including controls) were filled with 0.1% ovalbumin for 24 h at 4°C. The plate was washed and tapped dry. Duplicate samples of unknown or standard were added to DNP-ovalbumin and control wells, and these were allowed to stand for at least 6 h before being aspirated and the plate tapped dry. Fluids to be compared (e.g. serum and epididymal fluid from the same animal) were always run on the same plate to avoid plate-to-plate variation. Iodinated *Staphylococcus* protein A was then added to all wells and allowed to stand for at least 6 h, before being removed and the plate thoroughly washed and tapped dry. Individual wells were then cut from the plate and counted for duplicate 60-sec intervals in a gamma counter. Values for the unknown samples were extrapolated from a standard curve constructed from a series of standard Ig anti-DNP values. Times for equilibration and concentrations for saturation of anti-DNP and *Staphylococcus* protein A were independently determined by individual experiments. Because *Staphylococcus* protein A binds most strongly to IgG among the Ig classes of the rabbit (Goding, 1978), and *Staphylococcus* protein A was used as the radioactive label, most of the anti-DNP measured must necessarily have been of the IgG class.

Collection of epididymal fluids

Normal males (Groups A). Adults immunized and control non-immunized animals were anaesthetized with pentobarbitone sodium, blood was collected to exhaustion by cardiac puncture and the serum obtained was frozen at –20°C. The epididymis and vas deferens were immediately exposed, and the terminal corrugated segment of the cauda epididymidis that merges with the vas deferens was rapidly cannulated with a blunted 18- or 20-gauge needle inserted in a retrograde direction. The cannulation was performed under a dissecting microscope, expressly avoiding small vessels in the duct wall. The needle was connected to thin polyethylene tubing filled with red castor oil and attached to a peristaltic pump (Multistatic pump, Buchler). The progress of the oil could be observed easily through the wall of the epididymal tubule under a dissecting microscope. After a period of 10–15 min, the serosa was removed from a small area of the surface of the proximal cauda epididymidis, and after careful rinsing to avoid contamination, one protruding loop of epididymal tubule was incised discretely with microscissors under a dissecting microscope. The incision was made at about the level of section 8a (Jones, Hamilton & Fawcett, 1979) and epididymal content was collected smoothly with a mouth-operated micropipette (Clay-Adams) of 20, 50 or 100 µl according to the occasion. The content was centrifuged for 6–10 min in a Beckman microfuge, and the clear epididymal fluid separating from the sperm pellet was frozen at –20°C until assayed for specific anti-DNP Ig. Tissue samples were taken from the cauda and prepared for histological study to establish that the pressure of the oil had not caused rupture of the epithelium.

In addition to epididymal fluids, cerebro-spinal fluid was obtained from immunized males for comparison of the blood-brain and blood-epididymal barriers to IgG. The fluid was obtained by puncture of the cisterna magna immediately after death by exsanguination. If there was contamination with blood the fluid was discarded. However, clear, cell-free fluid was obtained in this way from 4 immunized males.

Experimental males. Epididymal fluid was also collected some 6 weeks after immunization from males whose reproductive tract had been manipulated in one of three ways. In Group B, the epididymis was exposed to body temperature for 6 days by unilateral reflection of testis and epididymis to the abdomen (Bedford, 1978). In Group C1, one testis was removed 6 days before epididymal fluid was collected, leaving the ipsilateral epididymis in the scrotum; and in Group C2, both testes were removed via a midline incision and both epididymides were replaced in the scrotum. When the testis was removed, particular care was taken to stem vascular oozing from the severed mesorchial vessels since bleeding would compromise scrotal cooling of the epididymis. In Group D, males were vasectomized bilaterally and then left for 3–4 months before epididymal fluid collection some 6 weeks after immunization. Because spermatozoa and fluid generally accumulate in and distend the rabbit duct for at least 6 months before any rupture ensues (Bedford, 1976; Moore & Bedford, 1978), it was possible to collect 1 ml of fluid without contamination from the vas deferens and at least 200 μ l from each epididymis of the vasectomized males. There was one exception in which vasectomy had been followed by reduction in testicular size and by obvious histological change in the seminiferous epithelium. In this male, numbers of unidentified cells were observed histologically in the proximal part of the epididymis.

Results

Thirteen rabbits that showed an immunological response to DNP–BGG were used to measure the absolute levels of that specific IgG in serum and in cauda epididymal fluid for each animal, and to establish the concentration ratio between the compartments. Data for this normal group and subsequent groups were derived from a standard curve such as that in Text-fig. 1, a separate curve being generated for each RIA plate. No activity was present in serum or epididymal fluid collected from 3 pre-immune males; therefore any activity measured was specific for anti-DNP–BGG IgG. The figures really reflected antibody binding directed against the DNP determinant since the wells of the plates were coated with DNP–ovalbumin.

Text-fig. 1. Standard curve used for the calibration of the solid-phase radioimmunoassay for anti-DNP IgG.

There was some variation in the antibody response of different animals, consistent with previous observation for rabbits (Eichmann, Braun & Krause, 1971). The absolute concentration of anti-DNP-BGG IgG measured in the serum of immunized males ranged from 1.3 to 26.0×10^{-5} M (mean 6.0×10^{-5} M). Thus this system of boosting and bleeding often evoked a high level of specific IgG. The present assay detected low and high affinity antibody, and comparable or greater specific responses have been reported with somewhat less sensitive methods to these and other antigens (Siskind, Dunn & Walter, 1968; Eichmann *et al.*, 1971). As expected, the level in epididymal fluid was much lower and varied, generally as a function of that of the serum concentration reflected above; the molar ratio of the concentration in epididymal fluid versus that in serum ranged from 2.5 to 11.0×10^{-3} (mean: 4.0×10^{-3}). Somewhat lower ratios were found for anti-DNP-BGG IgG in cerebrospinal fluid from 4 animals (0.75 – 2.2×10^{-3} ; mean: 1.7×10^{-3}).

The mean concentration of anti-DNP-BGG in fluid from the cauda epididymidis of all normal males was 3.8×10^{-7} mol/l (or 61.0 μ g/ml). Since it has been feasible to collect up to 100 μ l fluid per cauda on occasion, and there are about 5×10^8 spermatozoa per cauda in the rabbit, this equals about 1.0 μ g specific IgG/ 10^8 spermatozoa there. Since 1 μ g antibody = 10^{-6} g, and contains $10^{-6} \times (6.23 \times 10^{23} \text{ Avogadro's number}) / (1.6 \times 10^5 \text{ g mol.wt})$ or 4.0×10^{12} molecules of IgG, a response at the mean level of that found here to DNP-BGG provides about 4×10^{-12} molecules/ 10^8 spermatozoa, or some 40 000 specific IgG molecules/sperm cell.

To establish whether any significant portion of the IgG in epididymal fluid was being contributed by more proximal regions of the duct system, in 2 animals the levels were measured in fluids collected from the left and right cauda epididymidis. At 6 days after unilateral ligation of one corpus epididymidis the epididymal fluid/serum ratio on the control side was closely similar in both animals to that on the ligated side (19.0 and 20.0×10^{-3} and 4.0 and 6.1×10^{-3}). Since spermatozoa passively traverse most of the epididymis in this time (see Amann, 1981), this implies that the IgG measured in caudal fluids emanated locally in the cauda and was not contributed by more proximal regions of the duct system.

In studies to assess the effects of factors likely to alter the ratio seen in normal males (Table 1), the serum levels of anti-DNP-BGG fell within the range displayed by the normal group. However, in each of 3 males made unilaterally cryptepididymal 6 days previously, the molar ratio of specific IgG was higher in fluid from the cryptic cauda (range 13.0 – 95.0 ; means 49.0×10^{-3}) than in fluid from the sham-operated scrotal cauda (range 2.5 – 12.0 ; mean 6.8×10^{-3}), suggesting that the cauda epididymidis becomes more permeable to IgG under the influence of body temperature. On the other hand, the cauda epididymal fluid/serum ratio of IgG in 6 animals subjected to bilateral castration 6 days previously (range 2.9 – 19.0 ; mean 12.0×10^{-3}) was no different from that in the ipsilateral cauda of males castrated unilaterally 6 days earlier (range 8.6 – 19.0 ; mean 12.0×10^{-3}), and the means for both were only marginally greater than

Table 1. Epididymal fluid/serum specific IgG ratios in rabbits after different treatments

Group	Fluid Sample	No. of rabbits	Epididymal fluid IgG: serum IgG $\times 10^{-3}$	
			Mean	Range
A	Control cauda	13	4.0	2.5–11.0
B	Cryptepididymal cauda (6 days)	3	49.0	13.0–95.0
	Sham-operated contralateral cauda	3	6.8	2.5–12.0
C1	Unilateral castrate (6 days)	3	12.0	8.6–19.0
C2	Bilateral castrate (6 days)	6	12.0	2.9–19.0
D	Vasectomized cauda (3–4 months)	3	5.3	1.7–16.0

that for the normal group. It is possible that a longer period of androgen deprivation might result in a higher concentration of IgG in the epididymal compartment, but it was not feasible here to obtain sufficient fluid more than 6 days after removal of the ipsilateral testis. Finally, IgG levels were measured in epididymal fluid collected 3–4 months after vasectomy from 3 males, in all of which the epithelium of the distended duct still appeared intact. In these, the ratios of epididymal fluid IgG concentration versus that in serum varied from 1.7 to 16.0×10^{-3} (mean 5.3×10^{-3}), and did not differ from those expressed in normal males.

Discussion

This pilot study with a model antigen, DNP-BGG indicates that there is a distinct barrier to the passage of IgG from serum to the lumen of the rabbit epididymis. Nonetheless, the barrier does not exclude Ig, as had been supposed previously. In fact, the epididymis seems much more accessible to IgG than the testis. Total IgG in rete testis fluid is of the order of 0.04 mg/ml in normal rams (Johnson & Setchell, 1968), and so approximates the 0.061 mg/ml of specific anti-DNP IgG found here in caudal fluid. Assuming that specific antibody might represent $\sim 10\%$ of the total, there would be a total IgG level of about 0.6 mg/ml in caudal fluid. This accords with independent preliminary observation made with a specific sensitive immunoabsorbent assay (ELISA) of a range of 0.83 – 1.13 mg/ml total IgG in epididymal fluid from normal rabbits (S. S. Witkin & J. M. Bedford, unpublished results), confirming that the IgG is secreted in the epididymis and does not come from the testis.

In general, the level of specific IgG appearing in epididymal fluid seemed fixed in a ratio that tended to be a function of its concentration in serum. Since the mean molar ratio for cerebrospinal fluid serum of 1.7×10^{-3} was less than half that for epididymal fluid/serum, it appears that specific IgG may reach the lumen of the cauda epididymidis more easily than it crosses the blood–brain barrier. Nothing is known yet of the distribution of other Ig classes among these compartments of the reproductive tract. Since much of the sperm-agglutinating antibody in human semen is of the IgA class (Friberg, 1974), it would be of interest to determine how epididymal levels of IgA compare to those for IgG reported here.

The present studies suggest that the epididymal barrier can be perturbed, but not easily. It was unexpected that castration had so little effect since luminally perfused horseradish peroxidase was present between the epithelial cells in the cauda epididymidis of the rat castrated 14 days previously (Moore & Bedford, 1979). The 6 days elapsing here may have been too short a time for such an effect to appear. Subjection of the cauda to body temperature was followed by greater concentrations of specific IgG in the lumen, and this may bear on the situation in man because clothing raises the scrotal temperature by about 4°C (Ehrenberg, Ehrenstein & Hedgran, 1957; J. M. Bedford & M. Berrios, unpublished observations). The typical reaction of the rabbit cauda epididymidis/vas deferens in the first months after vasectomy, distension without duct rupture (Bedford, 1976; Moore & Bedford, 1978), did not bring increased passage of IgG to the duct lumen. Agglutinating antibody is present in epididymal fluids from men vasectomized for more than 8 months (Linnet & Fogh-Anderson, 1979), but it is likely that some focal rupture of the epididymal/vas epithelium with leucocyte invasion had occurred by that time (Alexander, 1972; Bedford, 1976; Pardanani, Patil & Pawar, 1976). Indeed, in 1 rabbit here (unreported) an abnormal response to vasectomy at 3 months, characterized by disturbance of spermatogenesis and, histologically, many cells in the proximal region of the epididymis, was associated with very high levels of specific IgG in the caudal fluids.

The finding of specific IgG in the epididymal compartment is of interest for two reasons. The epididymis secretes several components of unknown significance for sperm maturation and sperm storage (see Brooks, 1979; Hinton, 1980), including glycoproteins not present in serum (Killian & Amann, 1973; Jones & Dott, 1980; Voglmayr, Fairbanks, Jackowitz & Colella,

1980). The role of one or other might be clarified and sperm maturation might even be disrupted, if specific antibody against them could be introduced to the epididymal lumen at a sufficient concentration. The DNP measured here is strongly antigenic in rabbits, when coupled to a foreign protein, but whether specific epididymal components are or can be made appropriately antigenic for the homologous system is at present unknown. Clearly, the end results would depend not only on the antigenicity of the component in question, but also on the affinity of the antibody evoked. There is also the question of the availability of complement which potentiates the action of IgG by damaging the sperm head membranes (Bedford, 1970; Russo, Metz & Dunbar, 1976). It is unknown whether haemolytic complement exists in epididymal fluid, but C3, an essential component in either pathway of complement activation, is detectable in epididymal fluid by immunoassay at levels of 0.23–0.31 mg/ml, or about one quarter the concentration found in rabbit serum (S. S. Witkin & J. M. Bedford, unpublished results).

In fact, circulating antibody to spermatozoa does not necessarily affect fertility. There is a natural anti-sperm IgG in the serum of normal males (Chang, 1947; Johnson, 1973; Hancock, 1976, 1979) and although the presence of induced antibody may be correlated with infertility or sub-fertility (Rumke, Van Amstil, Messer & Bezemer, 1974; Husted, 1975; Alexander, 1977) some males with easily detectable serum levels remain fertile. However, nothing appears to be known about the molar concentration of circulating antibody in any of these men. The effects of Ig directed against red cells are exerted at levels of 20–20 000 molecules Ig/cell, according to the effect (Rosse, 1974; Logue & Rosse, 1976; Frank, Schreiber, Atkinson & Jaffe, 1977). Whether the present levels of anti-DNP-BGG of 40 000 molecules/spermatozoon would affect fertility at the epididymal level if directed against some element essential for sperm function remains to be determined.

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