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Restricted Entry of IgG into Male and Female Rabbit Reproductive Ducts Following Immunization with Recombinant Rabbit PH-20

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PROBLEM: Successful immunocontraception using sperm antigens is dependent on achieving sufficient sperm-specific antibody in the reproductive ducts to prevent fertilization. The blood : luminal barrier of the male and female reproductive ducts must be overcome for this to occur. We have, therefore, investigated the relative titers of antigen-specific immunoglobulin G (IgG) in luminal fluids collected from male and female rabbit reproductive ducts following immunization with recombinant rabbit PH-20 (rPH-20).

METHOD OF STUDY: Male and female rabbits were immunized subcutaneously with rPH-20 in Freund's adjuvant. Reproductive tract fluids and plasma were collected and assayed for specific IgG by enzyme-linked immunoabsorbent assay (ELISA). Plasma anti-rPH-20 antibodies were tested for their ability to inhibit *in vitro* fertilization.

RESULTS: Plasma rPH-20-specific IgG titers of $>21 \times 10^6$ were induced in bucks. Antibody levels in the rete testis and cauda epididymidis fluids were only 0.026 and 0.168% of plasma levels, respectively. Plasma IgG titers were $>30 \times 10^6$ in does, but antibody levels in free flow vaginal fluid, free flow uterine fluid and free flow oviduct fluid were only 0.016, 0.078 and 0.072% of plasma levels, respectively. Induction of ovulation by administration of human chorionic gonadotrophin (hCG) significantly increased rPH-20-specific IgG only in free flow vaginal fluids. Plasma antibody from immunized rabbits inhibited *in vitro* fertilization but conception rates following mating of rPH-20 immunized male to untreated female rabbits were not affected.

CONCLUSIONS: The IgG antibody entry into the reproductive ducts of both male and female rabbits is restricted to less than 0.2% of levels induced in plasma following subcutaneous immunization. This finding raises doubts about the suitability of rPH-20 to induce immunocontraception in rabbits using strategies optimized for induction of a serum antibody response. Whether mucosal immunization strategies can achieve this remains to be tested.

Key words:
Immunocontraception, immunoglobulin, rabbit, rPH-20

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INTRODUCTION

The introduction of feral rabbits into Australia has been an ecological disaster that has resulted in major environmental degradation, extinction of 18 species of Australian mammals and the endangerment of many more native animal species. Following the introduction of 24 founding animals the population of the European rabbit (*Oryctolagus cuniculus*) increased to almost 600 million prior to the release of the myxoma virus in the 1950s. This virus caused a rapid population crash because of the high lethality of the original virus (99.9%). However, within 3 years of release, attenuated strains of virus were isolated from the field and genetic resistance to infection developed rapidly in the rabbit population. As a result, rabbits are again a major problem and today cause an estimated $600 million annual loss in agricultural production. In the search for new methods to control rabbit populations fertility control may provide a method that is both effective and humane.[1]

Antibodies against spermatozoa can easily be induced (in plasma) in many animal species by immunization with sperm in adjuvant. In addition, there is substantial clinical literature associating human male infertility with the presence of antisperm antibodies. These observations have led to considerable interest in the development of immunocontraceptive vaccines as one possible means of controlling rabbits, and other feral pest species. Success of such a vaccine would require entry of sufficient antibody, directed at appropriate sperm surface antigens, into the male and/or female reproductive ducts to prevent fertilization. The ease with which such antibodies can be induced against even autologous sperm is because of the fact that sperm production does not begin until puberty[2] long after immune tolerance to self has been established. However, the fact that the majority of males do not develop antibody against sperm suggests the presence of mechanisms that prevent such autoimmune reactivity from occurring.

Mammalian spermatozoa that leave the testis must undergo several successive biochemical transformations during their transit through the epididymis before they acquire the ability to recognize and fertilize an oocyte. The epididymis provides the specific environment for sperm maturation and several epididymal proteins associate with sperm during the maturation process. Such proteins should make ideal immunocontraceptive antigens as immune responses against these antigens should disrupt sperm maturation but not compromise testicular spermatogenesis. Such an approach should result in infertility that is reversible. The validity of this approach has received some support from studies in mice where passive immunization with a polyclonal antibody directed against a 26-kDa epididymal sperm protein resulted in reversible infertility.[3,4] Although milligram amounts of antibody were required to cause infertility and this was only short-lived, this study did demonstrate, that rabbit IgG could enter the mouse epididymis in sufficient quantities to affect sperm development. Other studies, however, have suggested that epididymal proteins may be poor candidates for immunocontraceptive antigens because they do not act as auto- or alloantigens and are only able to elicit antibody production in a xenogeneic setting.[5]

Immunization of both male and female guinea-pigs with the sperm antigen rPH-20 has been shown to elicit infertility[6,7] suggesting that in this species antibody against sperm antigens can access both the male and female reproductive tracts (FRTs). However, immunization of male guinea-pigs with PH-20 was shown to elicit experimental allergic orchitis (EAO).[8] Thus, the permeability of the blood : luminal barrier may have been compromised by the inflammatory response associated with EAO and infertility may have been caused by both antibody and cell-mediated anti-PH-20 responses.

In the present study, we have immunized both male and female rabbits with rabbit rPH-20 produced in *Escherichia coli*.[9] We measured rPH-20-specific immunoglobulin G (IgG) in plasma and reproductive tract fluids in order to determine if plasma-derived antibody can access the male and FRTs in sufficient quantities to prevent fertilization.

MATERIALS AND METHODS

Animals

Male and female New Zealand White rabbits (> 6 months old weighting 3.5–5 kg) were obtained from Tillside Rabbit Stud (Luddenham, NSW, Australia) and were kept in separate rooms on a 12-hr light/dark cycle. Animals were provided with food and water *ad libitum*. All procedures were approved by the University of Newcastle Animal Care and Ethics Committee and the CSIRO Animal Care and Ethics Committee.

Immunization

Rabbit rPH-20 was prepared by CSIRO Sustainable Ecosystems (Canberra, Australia)[9]. Briefly, the rabbit PH-20 gene was cloned from a lambda gt11 library using guinea-pig PH-20 complementary DNA (cDNA) as a probe. The rabbit PH-20 gene was expressed in the FLAG system (Sigma, St Louis, MO, USA) to produce recombinant rabbit PH-20. An amount of 100 μg of

rPH-20 in 1 mL of saline was emulsified in 1 mL of complete Freund's adjuvant. Each animal was immunized subcutaneously with 0.2 mL of rPH-20 in adjuvant distributed over five injection sites. Two booster immunizations were performed in identical manner except that rPH-20 was emulsified in incomplete adjuvant. Animals were immunized three times over an 8-week period. Ten days after the final immunization, luminal fluids were collected.

Immunostaining of Rabbit Sperm

Rabbit spermatozoa from the cauda epididymis were collected by reverse flushing the vas deferens with phosphate-buffered saline (PBS). Sperm were collected by centrifugation at 750 ***g*** at room temperature, diluted in PBS and counted with a haemocytometer. Approximately 50,000 sperm were applied to a slide in 100 μL of PBS. Sperm were spread in a circle about 1.5 cm in diameter and allowed to air dry. Sperm were blocked overnight at room temperature with 3% (w/v) bovine serum albumin in PBS. The slides were washed in PBS and incubated for 1.5 hr at 37°C with mouse anti-rabbit rPH-20 sera from female BALB/c mice hyperimmunized with rPH-20, diluted 1 : 10 in PBS. The sperm were washed and incubated for 3 hr at 37°C with fluorescein isothiocyanate (FITC) conjugated goat anti-mouse serum (Silenius, Australia, at a dilution of 1 : 60) before being photographed under a confocal microscope.

Induction of Ovulation

One group of female rabbits were administered 50 IU of human chorionic gonadotrophin (hCG) into the lateral ear vein 12–14 hr prior to collection of luminal fluids. The occurrence of ovulation in the animals was confirmed during surgery.

Collection of Male Reproductive Tract Fluids

Rabbits were anaesthetized with sodium pentobarbitone (Nembutal, Abbot Laboratories, Sydney, NSW, Australia) by i.v. injection into the marginal ear vein. Following tracheotomy a breathing tube was inserted into the trachea. Animals were then maintained at 36–37°C on a heated table and viewed with an operating microscope (OpMi-1, Zeiss, Oberkochen, Germany) for the collection of the reproductive fluids. Fluid from the rete testis was collected by micropuncture using a micromanipulator (Leitz, Wetzlar, Germany), and micropuncture pipettes prepared from constant-bore glass tubing (i.d. = 70 μm, o.d. = 860 μm) using a Narashinge PN3 glass micropipette puller (Narashinge Scientific Instrument Laboratory, Tokyo).[10,11] Luminal fluid from the cauda epididymidis was collected into haematocrit tubes by cannulating the vas deferens and backflushing the distal cauda epididymidis with water-saturated paraffin oil. Samples of luminal fluid were diluted in 3% w/v sodium chloride and the concentration of sperm determined using a haemocytometer. The tubes containing the luminal fluids were sealed (Seal-ease, Becton Dickinson, Rutherford, NJ, USA) and the plasma was separated from sperm by centrifugation. The volume of luminal plasma was recorded before it was diluted in 100 μL of borate-buffered saline (BBS) containing 0.4 μg per μL phenylmethylsulphonyl fluoride (PMSF) (Sigma) and stored at –80°C.

Collection of Female Reproductive Tract Fluids

Samples were collected by two methods. Free-flow samples of luminal fluid were collected into cannulas. Vinyl tubing (i.d. = 0.4 mm; o.d. = 0.8 mm) was inserted through the infundibulum into the proximal end of the oviduct, and polyethylene tubing (i.d. = 0.5 mm; o.d. = 1.0 mm) was inserted through the vagina and cervix into the uterine lumen, and through the external opening into the vagina. The second method involved flushing 1 mL of BBS containing PMSF through the oviduct, uterus or vagina separately after the animal was euthanized by an overdose of Nembutal.[12] The free-flow fluids were diluted in the BBS containing PMSF and frozen as described above for the fluids from the males.

Reabsorption of Fluid and Antibody

Sperm were used as a volume marker to calculate the net percentage of fluid and rPH-20-specific antibody that was reabsorbed between the rete testis and cauda epididymidis of the male reproductive tract.[13]

ELISA Assay for Detection of rPH-20-specific Antibody

Wells of flexible 96-well microtiter plates (Falcon Immunoassay Plates, Becton Dickinson, Oxnard, CA, USA) were coated overnight at 4°C with rPH-20 (10 μg/mL in BBS, 50 μL per well). Plates were washed 5X with PBS then blocked for 2 hr at 37°C by addition of 200 μL per well of PBS containing 5% foetal calf serum (FCS) (TRACE Biosciences, Sydney, NSW, Australia). Plates were again washed 5X with PBS containing 5% Tween-20 (Sigma; PBS-T) and 50 μL/well of samples (diluted twofold in PBS-T) added. Plates were incubated for a further 2 hr at 37°C then washed 5X with PBS-T. About 50 μL/well of goat anti-rabbit IgG (diluted 1 : 1000, Southern Biotechnology Associates, Birmingham, AL, USA) was added and plates incubated overnight at 4°C. After further washing (5X, PBS-T) 50 μL/well of streptavidin-conjugated horseradish peroxidase (diluted 1 : 500; Amersham, Sydney, NSW, Australia) was added and plates incubated for a further hour at 37°C. After a final

wash (5X, PBS) 50 μL/well of tetramethylbenzidine substrate (TMB, Sigma) was added. After 15 min, colour development was stopped by addition of H_2SO_4 and plates were read at 450 and 695 nm.

In Vitro *Fertilization*

Mature domestic does were induced to superovulate by a single injection of 150 IU of pregnant mare's plasma gonadotrophin followed 60–72 hr later by 150 IU of hCG given i.v. Some 14 hr post-hCG administration the animals were killed by i.v. barbiturate overdose and the reproductive tract removed to ice. Oocytes were recovered from the oviduct by flushing with 3–5 mL of Brackett's medium (112 mM sodium chloride, 4 mM potassium chloride, 2.25 mM calcium chloride, 0.85 mM potassium dihydrogen phosphate, 37 mM sodium bicarbonate, 0.052 mM magnesium chloride, 14 mM glucose, 1.25 mM sodium pyruvate 0.3% (w/v) bovine plasma albumin and 0.003% (w/v) sodium penicillin pH 7.8). The oocytes were washed twice in Brackett's medium, treated with hyaluronidase (1 mg/mL) for 10 min to remove cumulus cells, washed again in Brackett's medium and placed into micro drops (100 μL) of Brackett's medium in Petri dishes under mineral oil thoroughly equilibrated in 5% carbon dioxide in air and incubated at 37°C.

Capacitated sperm were obtained by flushing the uterus of a doe 15 hr post-coitus with 4 mL of Brackett's medium equilibrated in 5% carbon dioxide in air and maintained at 37°C. Sperm were diluted in Brackett's medium to a final concentration of 5×10^6/mL. The sperm were incubated in rPH-20 antiserum diluted 1/20 in Brackett's medium for 30 min at 37°C. A 10-μL aliquot (5×10^4) was removed, mixed with the oocytes and the coculture incubated in 5% carbon dioxide in air at 37°C for 24–29 hr. The oocytes were removed to fresh medium, repeatedly pipetted up and down in a wide bore pipette to remove loosely bound sperm, and then placed on clean glass slides in 30–40 μL droplets of medium. A coverslip supported by paraffin wax at each corner was gently lowered onto the droplet and sealed in place with mineral oil to prevent drying out. Sperm and eggs were examined using inverted Normaski optics (Zeiss Model 9900; Carl Zeiss, Sydney, NSW, Australia) with a compound microscope. The number of sperm bound to the zona or present within the perivitelline space were counted, and the number of fertilized eggs and state of cleavage were recorded.

RESULTS

Despite identical immunization protocols, plasma rPH-20-specific IgG levels in female rabbit plasma were significantly higher than levels seen in male plasma (Fig. 1). Immunofluoresence using mouse antisera to rabbit rPH-20 localized PH-20 to the acrosome of sperm, mainly to inner acrosomal membrane and acrosomal contents (Fig. 2). It also reacted with testis sections in a specific manner and reacted with native PH-20 on Western blots (data not shown). Incubation of capacitated rabbit sperm for 30 min in a 1/20 dilution of serum from rPH-20 immunized rabbits, prior to incubation with rabbit oocytes, resulted in a 50–55% reduction in the number of fertilized eggs

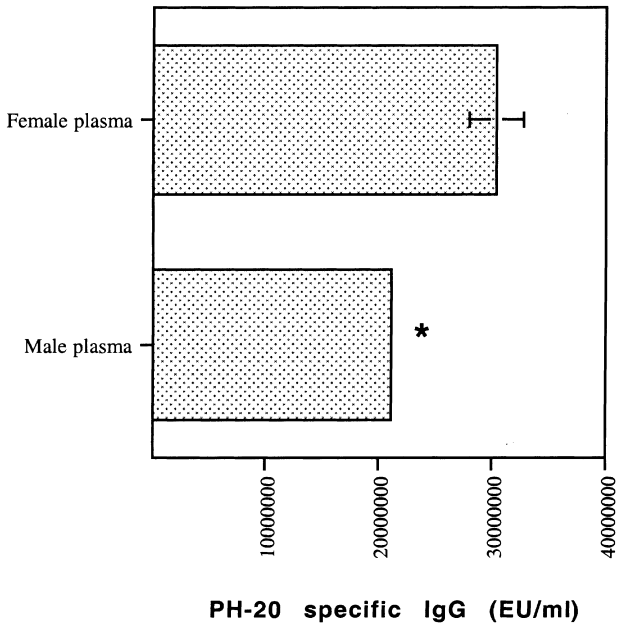

Fig. 1. The rPH-20-specific IgG in male and female plasma. Male and female rabbits were immunized with rPH-20 in Freund's adjuvant as outlined in Materials and Methods. Antibody titers were determined by ELISA. Data represent the mean antibody levels from groups of 5–6 animals.

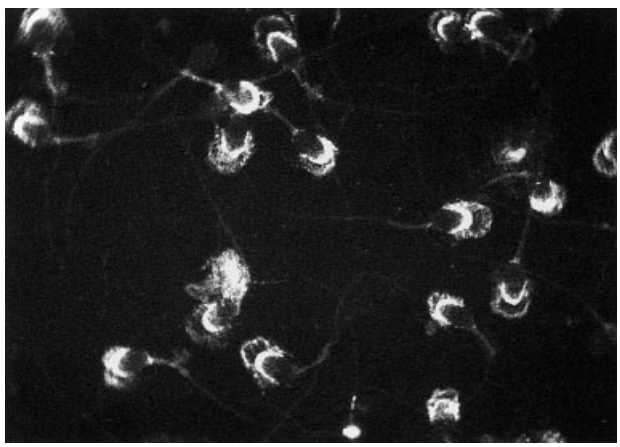

Fig. 2. Serum IgG from mice immunized with rPH-20 reacts with native PH-20 on capacitated rabbit sperm.

TABLE I. Serum antibody from rPH-20 immunized rabbits inhibits *in vitro* fertilization

	Serum	Number of eggs	Number fertilized (Percent fertilized)	Mean (± sem) number of sperm bound per egg
Rabbit 1	Pre-immune	37	35 (95%)	17.8 ± 8.2
	Immune	38	19 (50%)	3.6 ± 2.1
Rabbit 2	Pre-immune	41	39 (95%)	21.1 ± 9.1
	Immune	39	17 (44%)	5.7 ± 3.3

(Table I), indicating that antisera from rabbits immunized with rPH-20 can inhibit fertilization *in vitro*.

The PH-20-specific IgG levels in male reproductive tract fluids are shown in Fig. 3. Despite a male plasma IgG titer in excess of 2×10^7 EU/mL the levels of IgG in fluids from the rete testis and cauda epididymidis were only 0.027 and 0.169%, respectively, of plasma antibody levels (Fig. 3). Data represent mean antibody levels from 5 to 6 animals per group. The antibody titer is significantly higher in the fluids from the cauda epididymis than the rete testis, probably because of the greater net reabsorption of fluid (99.4%) rather than specific antibody resorption (95.8%) between the two sites (calculations based on changes in sperm concentration). Histological examination of the reproductive tracts of immunized males showed no signs of orchitis

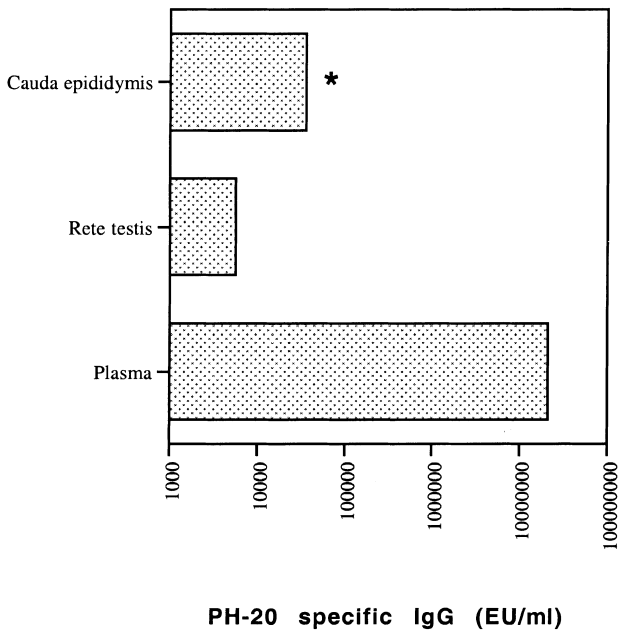

Fig. 3. The rPH-20-specific IgG in male plasma and reproductive ducts. Male rabbits were immunized with RPH-20 in Freund's adjuvant as outlined in Materials and Methods. Antibody titers in plasma or fluid from the cauda epididymis and rete testis were determined by ELISA. Standard error of the mean values was between 0.1 and 11%. Data represent the mean antibody levels from groups of 5–6 animals.

suggesting that the blood : luminal barrier had not been compromised as a result of inflammation (data not shown). These tissues were however collected 102 days after the first immunization by which time any inflammation may well have resolved.

Figure 4 shows that plasma-IgG entry into the FRT is also restricted. Female plasma titers were $> 30 \times 10^7$ EU/mL. However, free-flow samples of luminal fluid from the vagina, uterus and oviduct contained only 0.016, 0.078 and 0.072%, respectively, of plasma IgG levels. Antibody levels in fluids collected by flushing the reproductive tract were even lower than those in free-flow samples. The administration of hCG 12–14 hr prior to sample collection produced ovarian follicles that appeared ruptured on examination under the operating microscope. Figure 5 shows that the hCG administration enhanced rPH-20-specific IgG levels in free-flow fluid from the vagina >10-fold over non-hCG treated females ($P < 0.001$). However, antibody levels in plasma, uterus and oviducts were unaffected by hormone administration.

We have previously shown that female rabbits immunized with rPH-20 do not show reduced fertility when mated naturally.[9] In this experiment, we immunized six male rabbits, previously shown to be fertile, with rPH-20. All animals showed a strong antibody response in their serum. When these males were mated with non-immunized females, who had previously borne young, all females conceived and bore litters within the normal size range for the colony from which they were obtained (data not shown).

DISCUSSION

Immunization of rabbits with rabbit rPH-20 in Freund's adjuvant induced high levels of specific IgG in plasma as expected (Fig. 1). Interestingly, however, plasma anti-rPH20 antibody levels were higher in females than in males. Thus although the testis is an immunologically privileged site male animals respond less effectively to this sperm-associated antigen than do female rabbits raising the possibility of selective immunosuppression.

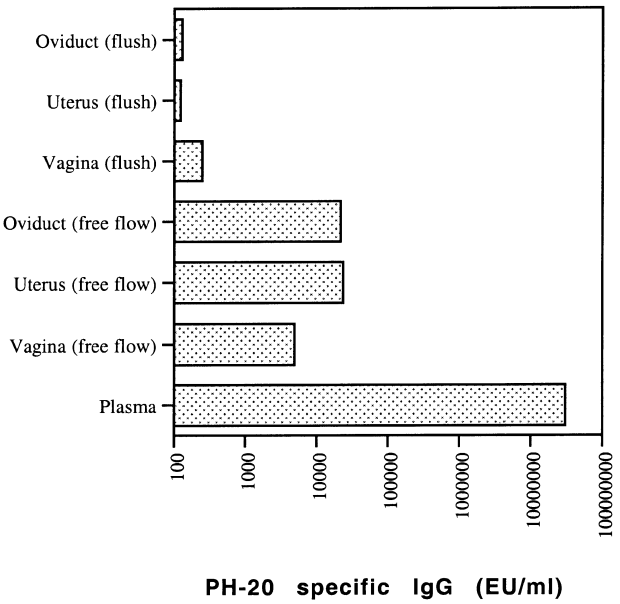

Fig. 4. The rPH-20-specific IgG in female plasma and reproductive tract fluids. Female rabbits were immunized with RPH-20 in Freund's adjuvant as outlined in Materials and Methods. Antibody titers in plasma and fluid from the vagina, uterus and oviduct were determined by ELISA. Standard error of the mean values was between 0.1 and 11%. Data represent the mean antibody levels from groups of 5–6 animals.

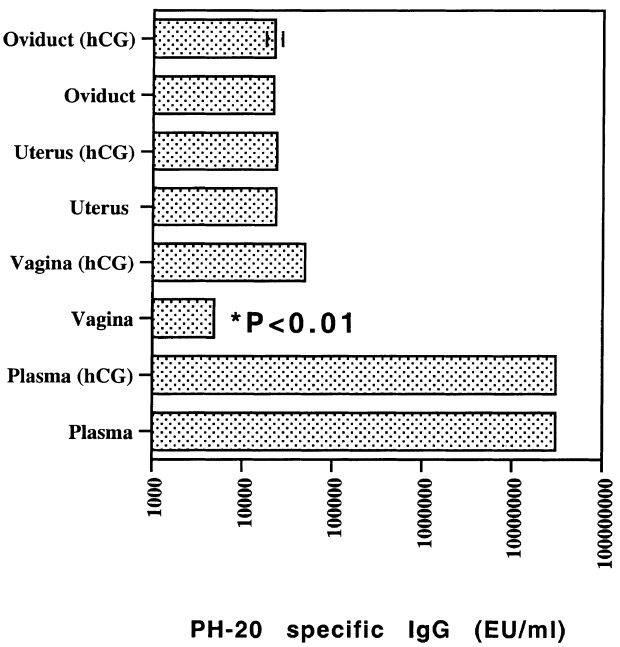

Fig. 5. Affect of hCG administration on rPH-20-specific IgG in female plasma and reproductive tract fluids. Female rabbits were immunized with rPH-20 in Freund's adjuvant as outlined in Materials and Methods. Prior to sample collection some animals were given hCG to induce ovulation. Antibody titers in plasma and fluid from vagina, uterus and oviduct were determined by ELISA. Data represent the mean antibody levels from groups of 5–6 animals.

The specific IgG in luminal samples from the male reproductive ducts were less than 0.2% of plasma antibody levels demonstrating that the blood–luminal barrier effectively prevents the entry of >99% of plasma antibody into the male reproductive ducts. This is consistent with the results of Weininger[14] who also demonstrated limited entry of IgG into rabbit male reproductive ducts. It is unlikely that IgG entered the seminiferous tubules as there is a significant barrier between blood and the lumen of the tubules.[15] This barrier is partly caused by the peritubular myoid cells that form a tubular tunic,[16] but is mainly the result of the specialized tight junctions between adjacent Sertoli cells which inhibit the passage of most solute, including lanthanum, into the lumen.[17] Although the barrier extends along the excurrent ducts of the testis, the juxtaluminal tight junctions between adjacent epithelial cells in the rete testis are not as well developed as the Sertoli–Sertoli cell junctions,[17,18] and the epithelium does not completely exclude small molecules. Consequently, the rete testis is probably the major site of entry of IgG from blood in rabbits (Fig. 3) as it is in the ram.[19] There is good evidence that the ductuli efferentes have a leaky epithelium to inorganic ions and water[20] but there is no evidence that it is permeable to protein. As the tight junctions in the epithelium lining the ductus epididymidis are well developed[21] and impermeable to most markers tested,[22] it is considered that there is probably no additional entry of IgG to the duct lumen via this route. The higher levels of IgG in fluid from the cauda epididymidis than the rete testis (Fig. 3) is probably because more fluid is reabsorbed (99.4%) than IgG is reabsorbed (95.8%) between the rete testis and cauda epididymidis. However, further work is required to confirm this conclusion. Sperm is also unlikely to be acting as a specific sink for IgG as the concentration of sperm in the cauda epididymidis was 150-fold that of the rete testis (Table II).

There is further evidence that the epididymal ducts are impermeable to the entry of IgG. Intravenous infusion of isotope or fluorochrome-labelled antibodies in a number of species has shown that antibody penetrates the extratubular interstitial tissues but does not enter the lumen.[23–25] Furthermore, *in vitro* studies involving incubation of segments of ductus epididymidis in solutions of labelled immunoglobulin indicate that antibody does not enter into the luminal compartment.[26] Other studies have, however, provided contradictory results.[27] Intravenous injection of anti-sperm antibodies into guinea-pigs resulted in the detection of antibody-coated spermatozoa in the rete testis and caput epididymidis, but not in the seminiferous tubules, within 30 min of injection. These data suggest that, in the guinea-pig at least, antibody can reach the

lumen in certain areas of the excurrent ducts. Whether these differences reflect true anatomical differences in the reproductive architecture between species or are artefacts of the different experimental approaches remains unresolved.

Entry of anti-rPH-20 IgG into the female reproductive ducts was also restricted with levels of rPH-20-specific IgG in free-flow fluid from the vagina, uterus and oviduct containing between 0.016 and 0.072% of plasma IgG levels (Fig. 4). Antibody levels in vaginal samples were significantly lower ($P < 0.01$) than in samples collected from the oviduct and uterus. It is difficult to attribute this difference to cyclical changes in oestrogen levels which differentially regulates antibody levels in rodent uterine and vaginal secretions[28] because the rabbit is an induced ovulator. To determine if ovulation in the rabbit is preceded by enhanced entry of antibody into the tract we pre-treated one group of female rabbits with hCG 12–14 hr prior to collection of reproductive tract samples. Antibody in vaginal samples was increased >10-fold in hCG-treated animals (Fig. 5) whilst there was no difference in anti-rPH-20 IgG content of uterine or oviduct secretions from non-treated versus hCG-treated animals. Why rabbits differ from rodents in this matter is unclear. However, although ovulation is associated with an increase in vascularization of the reproductive tract in the doe, it does not result in the bulk movement of fluid into the tract that is seen in rodents. It is tempting to speculate that in rodents antibody entry is merely a passive consequence of this bulk movement of fluid rather than a specific effect. Our data demonstrate that in the rabbit oviduct and uterus and to a lesser extent the vagina IgG movement from plasma into reproductive tract fluids is certainly restricted compared with what is seen in rodent models.

The immune system must protect against potential pathogens accessing the FRT without compromising conception or foetal survival. Nevertheless, the female may be exposed to allogeneic sperm or to a foetal-placental unit, both of which are foreign and have the potential to elicit an immune response. In order to meet the requirements for both immune protection and reproductive success, immunity in the FRT is tightly regulated by the female sex hormones to ensure both foetal and maternal survival (reviewed in[28]). Thus, depending on the site examined and the stage of the reproductive cycle, immunity in the female tract may be either enhanced or suppressed by the hormones oestrogen and progesterone. For example, IgG and IgA levels in rat uterine secretions are highest at the time of ovulation and treatment of ovariectomized rats with oestradiol increases antibody levels in uterine secretions compared with saline treated control animals.[29] Secretory component (SC)-mediated transport of IgA is also regulated by sex hormones. Estradiol treatment increases SC levels in rat uterine secretions but decreases levels in cervicovaginal secretions.[30] With regard to immunocontraceptive vaccines targeting sperm antigens, it will be essential to show that sex-hormone-mediated changes in rabbit reproductive tract immunity do not limit anti-sperm antibody availability at this site.

In the studies presented here we have clearly shown that entry of plasma IgG into rabbit reproductive ducts, both male and female, is restricted to less than 0.2% of plasma levels. Furthermore, immunization of rabbits with rPH-20 did not result in infertility in either males or females, despite the fact that plasma anti-rPH-20 antibody from both males and females reduced the numbers of ova fertilized *in vitro* (Table I). Serum antibody from mice immunized with rPH-20 also reacted with the region on rabbit sperm to which PH-20 is localized (Fig. 2), reacted with rabbit testis sections in a specific manner and bound to native PH-20 on Western blots (data not shown). Mouse anti-rPH-20 was used for staining of sperm (Fig. 2) in order to avoid problems of non-specific binding of the secondary anti-rabbit IgG reagent to rabbit IgG that may have associated with sperm. Subsequently, we have seen similar staining patterns when washed sperm were stained with serum from rPH-20 immunized rabbits (data not shown). Thus immunization resulted in plasma antibody that reacted with native PH-20 and could reduce fertilization *in vitro* but this antibody could not access the reproductive ducts of either males or females in sufficient quantities to mediate immunocontraception. An alternative explanation for the lack of effect of immunization on fertility could be that

TABLE II. Sperm concentration and anti rPH-20 IgG titer in male reproductive tract fluids

Sample	IgG titer (EU/ul)	Sperm concentration (x 10^3/ul)
Plasma	21,115,390 ± 26,249[a]	—
Rete testis fluid	5,598 ± 640[b]	34 ± 8
Cauda epididymis fluid	35,645 ± 3031[a]	5210 ± 249

a and b represent the means ± sem from 6 and 5 animals respectively.

PH-20 is not as important for sperm–egg binding in rabbits as it is in guinea-pigs. The distribution of PH-20 is certainly different between species. In the guinea-pig PH-20 is found predominantly on the post-acrosomal head region whilst in the rabbit it is found mainly in the acrosomal contents and the subacrosomal/perinuclear material (Fig. 2). Plasma from immunized rabbits, at a 1/20 dilution, only inhibited *in vitro* fertilization by 50% (Table I) whereas immunization of both male and female guinea-pigs with RPH-20 has been shown to elicit long lasting, reversible infertility.[6,7] Induction of ovulation in female rabbits did increase by >10-fold the levels of specific antibody in vaginal fluids, however, this still represented less than 0.2% of plasma IgG levels and is unlikely to be sufficient to prevent fertilization, particularly as sperm spend only a few minutes in the vagina after mating.[31] Our data suggest that, in the rabbit at least, immunization with reproductive antigens expressed only in the reproductive tract using routes which induce predominantly plasma IgG are unlikely to result in reduced fertility because antibody cannot access the reproductive ducts. Studies are presently in progress to determine if immunization methods that elicit mucosal immune responses may be more effective at inducing immunocontraception. Because rabbits are known to have at least 11 functional IgA genes[32] this approach may be more likely to work in this species. In addition, we are now focusing on using antigens accessible to plasma antibody, such as zona pelucida antigens, as the basis for our immunocontraceptive vaccine.

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