Influence of complement depletion on sperm function in the female rabbit

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Summary. Female rabbits were wholly depleted of complement by treatment with anti-complementary cobra venom factor (CVF) 36 h before mating. Complement depletion did not compromise occurrence of the acrosome reaction, as judged by sperm penetration of eggs collected 12–13 h *post coitum*. However, in CVF-treated females, significantly more spermatozoa had penetrated the egg vestments, more spermatozoa were present in flushings from the oviducts, and sometimes the uterus, than in control females mated to the same males. The results indicate that, although the acrosome reaction is unlikely to depend on complement activation, complement-dependent factors may exert a restrictive effect on spermatozoa after vaginal insemination of the normal female rabbit.

Introduction

Investigation of conception in mammals has been helped in the past two decades by development of reliable methods for superovulation, sperm collection, and in-vitro fertilization. There are still many unknowns, however, with respect to the factors that finally determine the functional state and fate of spermatozoa in the female tract. It is not clear what is the significance of the apparently excessive number of spermatozoa inseminated, how the tract reacts to antigens expressed by spermatozoa, what factors bring about capacitation there, or how sperm transport to the ampulla of the oviduct is regulated. It has also been unclear what regulates the acrosome reaction that spermatozoa must undergo in order to fertilize. In the invertebrates studied, the acrosome reaction is initiated by egg-coat substances which permit a calcium influx (Dan, 1954). There is no good evidence, however, that egg-derived factors must induce the acrosome reaction in eutherian mammals. It has seemed credible that other factors may regulate this in mammals, and as one possibility, it has been suggested that complement components found in follicular fluids may act at the sperm surface to initiate the membrane fusion that is the first step in the acrosome reaction (Cabot & Oliphant, 1978).

The idea that follicular fluid complement may initiate the acrosome reaction is somewhat weakened by the fact that follicular fluid is not required for fertilization *in vivo* (Harper, 1970; Moore & Bedford, 1978; Saling & Bedford, 1981). Complement in tubal secretions might play such a role, but there is no clear picture as to which components of the complement system are represented throughout the female tract of the rabbit (Oliphant, Randall & Cabot, 1977). However, the presence of antibody-coated rabbit ova in the oviduct evokes a mobilization of leucocytes which

coat the zona pellucida within 3 h, though generally not in rabbits previously given anticomplementary cobra venom factor (unpublished observations). Since leucocyte migration is probably a response to complement activation, this suggests that complement activation, via the classical pathway at least, can occur in the ampulla.

Based on the assumption that whole body depletion of complement is reflected in the lumen of the oviduct, we have used that approach to examine the role of complement activation in the process of fertilization.

Materials and Methods

New Zealand White rabbits weighing 3½–4 kg were used to determine the effects of complement depletion on fertilization and, as a result of initial observations, on sperm distribution in the female tract.

Complement depletion was achieved in the rabbits by intravenous injection of an anticomplementary factor (CVF) prepared from cobra (*Naja naja*) venom according to the procedure of Ballou & Cochrane (1969). Preliminary experiments using a haemolytic complement assay showed that any sample of 0.025–0.3 ml of the CVF preparation containing 6 mg protein/ml would deplete rabbit serum of complement by 12 h after intravenous injection, and for at least 5 days thereafter. A dose of 0.2 ml of that CVF preparation was used then throughout the subsequent study. Initial trials also showed that the fall in haemolytic complement was paralleled by a disappearance from the serum of a spermicidal effect (Chang, 1947) known to be produced by naturally occurring antisperm IgG in the presence of complement (Johnson, 1968; Hancock, 1979). The spermicidal effect is demonstrated easily by adding a dilute (50×10^3 /ml) suspension of motile ejaculated rabbit spermatozoa in Tyrode's solution (Tyrode, 1910) to an excess of serum, with examination in a phase-contrast microscope 2 min later. Independent assays performed using an enzyme-linked immunosorbent assay (ELISA) (Jackson, Sogn & Kindt, 1982) showed no change in total IgG levels in the serum 36–48 h after CVF administration. Therefore, the disappearance of serum spermicidal activity after treatment with CVF served as a useful spot test of the complement status in each animal.

The experimental protocols involved natural mating of females given 0.2 ml CVF 36 h previously, and also mating of a series of untreated (control) female rabbits to the same individual males. Each female was mated with 3 stud bucks of high semen quality, within a few minutes. The matings of experimental and control females were generally interspersed with each other. The males were not used more than once every 48 h. After mating, 50 i.u. hCG were given intravenously to ensure ovulation. The females were killed by cervical dislocation, and blood was withdrawn by heart puncture. The abdomen was immediately opened and haemostats were placed on each uterotubal junction. The uterine horns were then flushed with Tyrode's solution via the cervix; subsequently, this was repeated 3–4 times with 0.5% sodium dodecyl sulphate (SDS). The oviducts were flushed in both directions, first with Tyrode's solution and then with 0.5% SDS. Total numbers of spermatozoa in the tubal and uterine flushes were counted with a haemocytometer. Ova found in the first tubal flush were mounted immediately beneath wax-supported coverslips and examined in a phase-contrast microscope for numbers of perivitelline spermatozoa. When necessary, the follicular cells were removed first by treatment of eggs with 1% hyaluronidase (Sigma Type I) in Tyrode's solution and shaking in 2% sodium citrate. Counts were made of total numbers of spermatozoa within each perivitelline space. After fixation in acetic alcohol and staining in lacmoid, the eggs were also examined for evidence of penetration according to the presence of a sperm tail and sperm nucleus.

The results were evaluated statistically using the χ^2 test for numbers of ova ovulated and the proportion of ova penetrated. Differences in sperm numbers in the perivitelline space, uterus and oviduct were assessed using the non-parametric Mann–Whitney test.

Results

In the initial experiments, 2 normal females were injected intravenously with CVF, then mated with 3 males 36 h later and given hCG. The CVF-treated females seemed vigorous and healthy, and there was no reduction of the level of IgG in the serum as measured by an ELISA for rabbit immunoglobulin. Because serum taken at the time of death showed no significant haemolytic activity and no spermicidal activity, the females clearly were depleted of complement by the CVF. Eggs were flushed from the oviducts 14 h after mating. All had been penetrated (9/9 and 11/11), and between 6 and 25 perivitelline spermatozoa were present per egg. Furthermore, as judged by our previous experience, there appeared to be more spermatozoa swimming in the flushed medium around the mounted eggs than was usually seen in the course of such procedures. This preliminary result indicated that an absence of complement does not compromise sperm penetration of the eggs and so, by inference, occurrence of the acrosome reaction. However, since the findings gave the impression that complement depletion may have some influence on the distribution of spermatozoa in the female after mating, the study was extended to examine this aspect further.

Each of 14 females given CVF 36 h previously, and also 8 normal untreated females, were mated 3 times to the same 3 bucks. Among both groups at 12–13 h after hCG there was some variation in sperm numbers associated with the eggs recovered, and in the tract flushings, as might be expected. However, as shown in Table 1, in the CVF-treated females significantly more eggs

Table 1. Rates of egg penetration and sperm numbers in the uterus and oviducts, in untreated and complement-depleted female rabbits after mating with the same 3 bucks

Animal	Time after hCG (h)	Numbers of				
		Ova collected	Ova penetrated	Perivitelline sperm./ovum (mean)	Uterine sperm. $\times 10^6$	Tubal sperm. $\times 10^6$
<i>CVF-treated females</i>						
1	12	7	7	6	4.5	0.26
2	13.25	Ripe follicles—no ovulation				
3	13	13	13	14	7.8	0.96
4	12.5	8	8	3	3.6	0.42
5	13	3	3	20	10.4	0.32
6	12.5	5	5	32	3.8	0.68
7	13	9	9	11	4.2	0.35
8	12.5	Ripe follicles—no ovulation			3.2	0.16
9	13	4	4	9	4.3	0.40
10	12	8	8	11	8.2	0.25
11	12.5	12	7	15	3.4	0.21
12	12.5	2	2	30	12.8	0.40
13	13	Ripe follicles—no ovulation				
14	12.5	3	3	2.0	3.2	0.28
Mean		74	69*	12.6†	5.8‡	0.39§
<i>Untreated (control) females</i>						
1	13	9	9	0.3	3.2	0.04
2	12.5	5	2	1.5	2.4	0.092
3	13	13	3	0.1	0.6	0.024
4	12.5	11	2	0.2	2.9	0.006
5	13	9	7	6	7.5	0.072
6	13	7	7	8	5.0	0.025
7	12.5	9	3	0.2	1.0	0.003
8	12	2	0	0	0.9	0.01
Mean		65	33	2.0	2.9	0.038

Compared with values for control females: * $P < 0.005$ (χ^2), † $P \sim 0.002$ (Mann–Whitney), ‡ $P \sim 0.02$ (Mann–Whitney), § $P < 0.001$ (Mann–Whitney).

were penetrated at 12–13 h after hCG ($P < 0.005$), there were more perivitelline spermatozoa ($P \sim 0.002$) and more spermatozoa were flushed from the oviducts ($P < 0.001$). The number of spermatozoa recovered from the uterus at 12–13 h was also generally higher than that from the normal rabbits ($P \sim 0.02$).

The results were variable, however, from female to female. In Rabbit 12, both eggs from one oviduct were covered with many motile spermatozoa to the extent that they rotated on the glass slide before being anchored by pressure from the coverslip. In Rabbits 11 and 14, there were very few perivitelline spermatozoa and not all eggs of Rabbit 11 were penetrated. Three of the females had not ovulated, although ripe follicles were present and oocytes released by puncture of these follicles had extruded the first polar body. In Rabbit 5, ova were found adhering to the surface of the ovary in what seemed unusually viscous cumulus oophorus with two other ripe follicles unruptured.

There were also 2 aberrant females not included in Table 1. The serum of one showed normal spermicidal activity 48 h after CVF injection, but only 4/11 eggs were penetrated at 13 h after hCG with a mean of less than one perivitelline spermatozoon. Conversely, one control female had no spermicidal activity in her serum when killed 12 h after hCG, and added motile spermatozoa were agglutinated in it by their tail surfaces. At 12.5 h the 2 eggs recovered were penetrated with 8 and 10 perivitelline spermatozoa, respectively. Thus, on the basis of the spermicidal activity of their serum, both females followed the general trend set in the experimental and control groups.

Discussion

CVF administration, in addition to depleting complement for about 5 days (Ballow & Cochrane, 1969), can result in suppression of T cell-dependent antibody production (Martinelli, Matsuda & Osler, 1978) and induce other short-lived haemodynamic effects (Ulevitch & Cochrane, 1977). However, the present experiments were not performed until 36 h after CVF injection, thereby minimizing these other effects, as well as eliminating concern about a possible phospholipase A contaminant (Lachmann, Halbwachs, Gewurz & Gewurz, 1976). The absence of haemolytic complement activity and also the spermicidal activity of the sera of treated rabbits that is complement dependent (Chang, 1947; Hancock, 1979), strongly implicate complement depletion as the central consequence of CVF administration in these experiments.

Although human cervical mucus possesses haemolytic complement activity (Price & Boettcher, 1979), no complete picture exists as to the complement components at each level of the female tract (Oliphant *et al.*, 1977; Schumacher, 1980). However, because complement-free conditions are employed in a variety of in-vitro capacitation/fertilization studies in different species (see Rogers, 1978) and in the present study fertilization occurred more readily in females depleted of complement 36 h before insemination, it is unlikely that the acrosome reaction depends on complement components acting at the surface of the spermatozoon, as suggested by Cabot & Oliphant (1978). Although the acrosomal disruption induced by complement-dependent antibody may resemble the physiological acrosome reaction on occasion, this generally presents as a random breakdown of the peri-acrosomal plasma membrane, which may itself form vesicles, and of the outer acrosomal membrane (Bedford, 1969; Russo & Metz, 1974; Roomans & Afzelius, 1975). The available evidence raises an alternative possibility that the mammalian acrosome reaction is regulated in individual spermatozoa as a function of the process of capacitation (Bedford, 1983).

While complement may not be a key element in the onset of the acrosome reaction, our results suggest that complement-dependent factors play some role in sperm function in the female. The anticomplementary factors present in seminal plasma (Suarez & Oliphant, 1982) are probably removed along with other seminal plasma components from spermatozoa as they swim in the uterus (Oliphant & Brackett, 1973). It may therefore be important that the normal sperm surface can activate complement via the alternative pathway of the complement activation sequence (Clark & Klebanoff, 1976; Witkin, Richards & Bedford, 1983), because this suggests that the presence of

specific antibody is not required for spermatozoa to initiate the activation of complement in the female tract.

The transport of spermatozoa into the rabbit female tract after insemination is not a random process, but usually follows a pattern characterized by rapid transport of a few spermatozoa to the oviduct, an intervening phase of low numbers in the uterus and oviduct, and a final slow build-up beginning at 3–4 h in the uterus and about 6 h *post coitum* in the oviduct (Overstreet & Cooper, 1978). In complement-depleted females there were more spermatozoa than normal in the oviduct and often in the uterus, some 12–13 h after vaginal insemination. Complement depletion also resulted in a significantly higher proportion of eggs fertilized and in more spermatozoa in the perivitelline space of fertilized eggs than in normal females at 12–13 h after hCG. This implies that complement-mediated factors may normally exert some restrictive effect on spermatozoa in the female tract. This was also suggested in pilot studies performed for other reasons on rabbits with serum sickness (with B. Albini, B. K. Noble & G. A. Andres, Department of Microbiology, University of Buffalo). Although precise sperm counts were not made, 2 rabbits inseminated vaginally in the terminal stages of serum sickness and essentially complement-depleted gave a similar strong impression of greater-than-normal numbers of spermatozoa in flushings from the uterus and oviducts. In these animals also, the serum had lost the spermicidal activity typical of normal individuals.

It remains to be established why more spermatozoa are able to reach the oviducts in complement-depleted female rabbits. Possible complement-dependent mechanisms that may militate against spermatozoa in the normal female rabbit could involve naturally occurring antibodies and leucocytes. IgG is bound to many spermatozoa flushed from the normal uterus, particularly to dead spermatozoa (Symons, 1967). However, whether this compromises the function of living spermatozoa is not clear, since they may bind IgG non-specifically via its Fc portion (Allen & Bourne, 1978; Witkin, Shahani, Gupta, Good & Day, 1980; Sethi & Brandis, 1980), an interaction of unknown significance that would not result in complement fixation. Mammals also possess naturally occurring anti-sperm IgG in serum that does fix complement and kills spermatozoa by assembly of the membrane attack complex (Johnson, 1968; Hancock, 1979), but its transduction into the tract at significant levels is still questionable (McAnulty & Morton, 1978). Leucocytes and sperm phagocytosis have long been recognized as sequelae to insemination (Austin, 1957; Bedford, 1965), and in the rabbit leucocytes appear after insemination in the vagina (Phillips & Mahler, 1977), cervix (Tyler, 1977), and uterus (Soupart, 1970). A by-product of complement activation, the anaphylotoxin C5a, could be partly responsible for this leucocyte influx. It is easy to visualize that complement depletion may prevent leucocyte chemotaxis and their effects on spermatozoa. Arguing against leucocyte involvement as a main cause of the present results, however, are observations that the presence of leucocytes does not seem to compromise seriously the passage of spermatozoa through the cervical canal and on into the uterus (Taylor, 1982), and that leucocytes do not normally appear in the tubal lumen even after insemination. Moreover, spermatozoa are not the sole stimulus for the movement of leucocytes into the lumen of the non-infected tract. White cells invade the rodent uterus at the termination of oestrus whether or not spermatozoa are present (Austin, 1957), and they appear in the vagina after mating to vasectomized males (Phillips & Mahler, 1977).

In these studies, 3 of the 14 CVF-treated females had not ovulated and one other had not completed ovulation by 12·5–13·25 h after hCG. This is not unprecedented according to the normal distribution reported by Harper (1960), but the proportion is higher than might be expected, indicating that complement-related factors could perhaps influence the follicular events that lead to ovulation (Espey, 1980).

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