J. Reprod. Fert. (1984) **72**, 467–471

Printed in Great Britain

Antibodies against epididymal glycoproteins block fertilizing ability in rat

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Summary. Antiserum against rat androgen-dependent secretory epididymal protein DE (raised in rabbit) was added to suspensions of rat spermatozoa from the cauda epididymidis which were used for artificial insemination. While control spermatozoa fertilized 41·6% of oocytes, those exposed to antiserum to protein DE fertilized only 6·6% ($P < 0·01$). An equal amount of normal rabbit serum (NRS) did not cause inhibition (33·1%). To study the entry of antibodies into the epididymis, caudal tubules were cultured for 24 h and the fertility of the contained spermatozoa was assessed by artificial insemination. Culture in Medium 199 alone or with NRS resulted in spermatozoa which fertilized 52% of oocytes while the presence of antiserum to protein DE in the culture medium yielded spermatozoa which fertilized only 16·6% of oocytes ($P < 0·01$). These results suggest (1) that the epididymal protein DE might be part of a sperm structure involved in the fertilization process, and (2) that, at least under the present culture conditions, immunoglobulins penetrate the epididymal epithelium in sufficient numbers to reduce fertility significantly.

Introduction

A growing body of evidence substantiates the participation of epididymal secretory glycoproteins in the sperm surface modifications required for the acquisition of fertilizing ability during epididymal maturation (rat: Fournier-Delpech, Bayard & Boulard, 1973; Lea, Petrusz & French, 1978; Olson & Hamilton, 1978; Kohane, González Echeverría, Piñeiro & Blaquier, 1980b; rabbit and hamster; Moore, 1980, 1981; González Echeverría, Cuasnicú & Blaquier, 1982; bull: Vierula & Rajaniemi, 1980; ram: Voglmayr, Fairbanks, Jackowitz & Collela, 1980). We have stressed the possible role of these moieties in the development of zona pellucida binding ability and fertilizing ability by maturing hamster spermatozoa (Cuasnicú, González Echeverría, Piazza & Blaquier, 1984a; Cuasnicú, González Echeverría, Piazza, Piñeiro & Blaquier, 1984b; González Echeverría, Cuasnicú, Piazza, Piñeiro & Blaquier, 1984).

Rat protein DE (Garberi, Kohane, Cameo & Blaquier, 1979) was shown to be progressively added to the head of the spermatozoon during epididymal transit (Kohane *et al.*, 1980b) and is apparently the same moiety as acidic epididymal glycoprotein (AEG; Lea *et al.*, 1978), protein IV (Jones, Brown, von Glos & Parker, 1980), protein DE (Brooks & Higgins, 1980), sialoprotein (Faye, Duguet, Mazzuca & Bayard, 1980) and the 32K rat epididymal protein (Wong & Tsang, 1982). We have now studied the ability of a specific antibody against rat epididymal protein DE to block the fertility of mature rat spermatozoa.

The entry of immunoglobulins into the epididymal lumen remains controversial (Weininger, Fisher, Rifkin & Bedford, 1982; Wong, Tsang, Fu & Lau, 1983), and we have also investigated the ability of the immunoglobulins to permeate cultured epididymal tubules and interact with the contained spermatozoa.

Materials and Methods

Antiserum. Antiserum against rat androgen-dependent secretory epididymal protein DE (anti-DE) was raised in rabbits and characterized as described by Kohane, Piñeiro & Blaquier (1983). A gamma globulin-enriched fraction was prepared by precipitation at 45% saturation of ammonium sulphate and purified by DEAE cellulose chromatography as described by Fahey & Terry (1973). Preimmune normal rabbit serum (NRS), treated similarly, was used as control.

Animals. Adult (90–120 days old) male and immature (25–30 days old) female rats of the Wistar strain were used, and were kept with food and water *ad libitum* and in 14 h light/24 h.

Artificial insemination. Tubules of the cauda epididymidis were cut up in 3 ml prewarmed (37°C) BMOC balanced salt solution (Brinster, 1965) and the concentration of the released spermatozoa was adjusted to 1×10^6/ml. When required, antiserum to DE or NRS was added to the medium at a final concentration of 0·1 mg protein/ml. Female rats, superovulated by the sequential injection of 30 i.u. PMSG followed 48–72 h later by 50 i.u. hCG, were inseminated 9 h after hCG. Aliquants (0·1 ml) of sperm suspension were injected into the mid-segment of one uterine horn and a ligature was placed distal to the site of injection; the corresponding control sperm suspension was inseminated in the contralateral uterine horn. Care was taken to maintain constant temperature (37°C) throughout all these operations. Animals were killed 24–48 h later, the oviducts flushed with phosphate-buffered saline (PBS), pH 7·4, and the cumulus incubated for 5 min at room temperature in hyaluronidase (480 i.u./ml) prepared in 1% polyvinylpyrrolidone in PBS and then washed in PBS. Oocytes were considered fertilized when they contained two pronuclei and a sperm tail in the vitellus or were at the 2-cell stage.

Organ culture. Epididymides were removed under sterile conditions and the tubules of the caudal segment were dissected under magnification until partly unconvoluted and ligated at both ends to avoid sperm leakage. The tissues were placed in organ culture dishes, Medium 199 (GIBCO, New York, U.S.A., with Earle's salts), containing 100 i.u. penicillin/ml and 100 μg streptomycin/ml, was added and the incubation continued at 33°C with a gas phase of 95% O_2 and 5% CO_2. As indicated, NRS or anti-DE serum was added to the medium at a final concentration of 0·1 mg protein/ml. After washing the tissues, spermatozoa contained in the cultured tubules were recovered and used for artificial insemination as described above.

Immunocytochemical staining. The presence of antibodies to protein DE on spermatozoa was investigated using goat anti-rabbit IgG serum labelled with horseradish peroxidase (Polysciences Inc, Warrington, PA, U.S.A.). Sperm smears were air-dried, fixed for 10 min in 4% *p*-formaldehyde and washed twice in PBS. Smears were incubated in a moist chamber with anti-rabbit IgG (1 : 5) for 1 h at room temperature and washed three times with PBS. Peroxidase was visualized by incubation with 0·04% 3,3′-diaminobenzidine and 0·03% H_2O_2 for 7 min at room temperature.

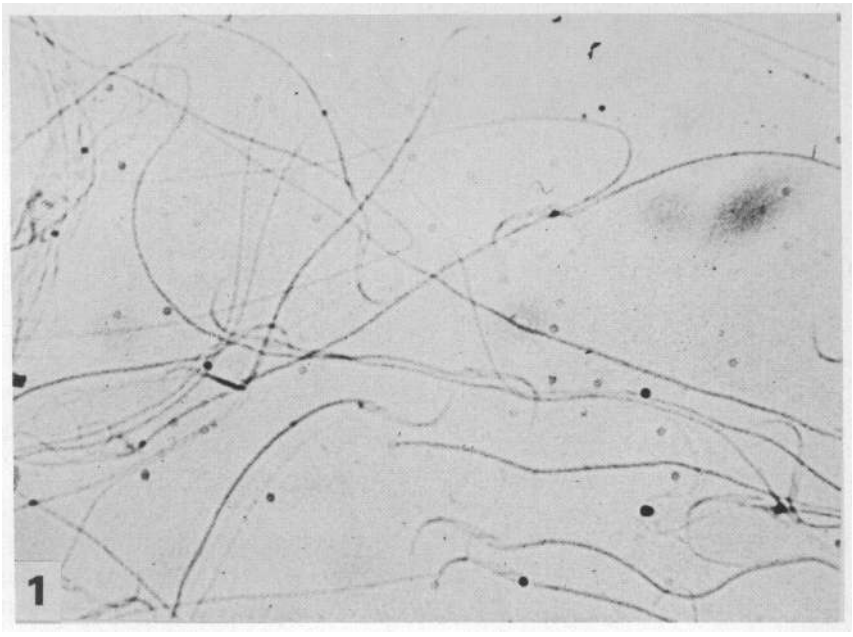
Statistical analysis. To avoid individual and interassay variations experiments were performed with paired samples in which the control and experimental sperm populations were obtained from the same animal. Results were analysed for statistical significance by the Mann–Whitney test.

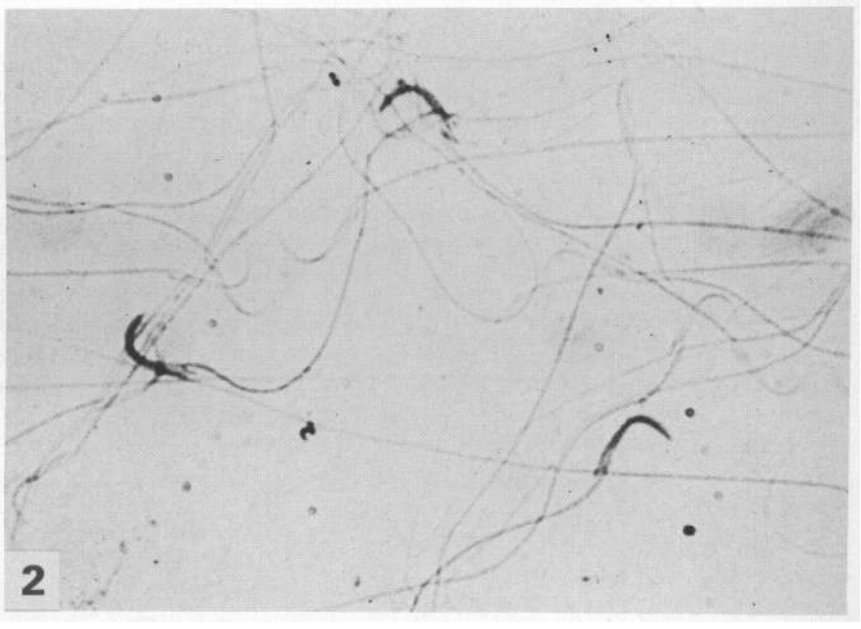
Results

The effect of antiserum to protein DE on sperm fertilizing capacity

Caudal spermatozoa were obtained from normal rats and suspended in Medium BMOC alone or containing NRS or anti-DE serum. A drop of these suspensions was examined by phase-contrast microscopy. No major change in the motility of the spermatozoa or evidence of agglutination was found in these preparations. The suspensions were used for artificial insemination within 10 min of preparation and the fertility rates obtained for each group are shown in Table 1. The results

PLATE 1





Immunocytochemical staining of spermatozoa recovered from tubules cultured in the presence of normal rabbit serum (Fig. 1) or anti-DE serum (Fig. 2).

(*Facing p.* 468)

Table 1. Effect of anti-DE serum or normal rabbit serum (NRS) on the fertilizing ability of rat spermatozoa from the cauda epididymidis tested by in-vivo insemination

Serum added	No. of observations	No. of oocytes fertilized/no. recovered	% Fertilization (mean ± s.e.m.)
None	8	63/147	41·6 ± 14·3
NRS	19	78/256	33·1 ± 7·3
Anti-DE	11	11/151	*6·6 ± 3·8

* $P < 0.01$ compared with values for the other two groups.

indicate that while the addition of NRS to the medium did not alter the fertilizing ability of spermatozoa, the presence of anti-DE significantly decreased fertility.

Penetration of antibodies to protein DE into cultured tubules and effect on fertility of the contained spermatozoa

To study the effect of culture conditions on the fertility of contained spermatozoa, one epididymis was removed and its caudal portion cultured as described in 'Materials and Methods' while the contralateral organ remained *in situ* and served as a control. A culture period of 24 h did not affect fertility since control spermatozoa fertilized 56·6 ± 9·6% (69/122) of oocytes while those from cultured tubules fertilized 49·3 ± 8·9% (39/80) oocytes (8 independent observations for both).

The system was then used to study the effect upon sperm fertility of NRS and anti-DE serum added to the medium during the 24-h culture period. The fertility of spermatozoa was not affected by NRS but was decreased significantly when antiserum to protein DE was present (Table 2). The motility of this sperm population was preserved at the level of control cultures. When examined by immunocytochemical staining, a totally negative reaction was obtained with spermatozoa recovered from control and NRS-exposed cultures while positively stained sperm heads (8·8%, 29/327 in two independent observations) were observed in samples obtained from the cultures with anti-DE serum (Pl. 1, Fig. 1).

Table 2. Effect of anti-DE serum or normal rabbit serum (NRS) added to the culture medium on the fertilizing ability of rat spermatozoa from the cauda epididymidis

Serum added	No. of observations	No. of oocytes fertilized/no. recovered	% Fertilization (mean ± s.e.m.)
None	9	42/80	52·6 ± 9·5
NRS	5	33/63	52·2 ± 14·1
Anti-DE	14	22/129	*16·6 ± 6·4

* $P < 0.01$ compared with values for other two groups.

Discussion

Our results demonstrate that the interaction of mature rat spermatozoa with antiserum to protein DE results in the loss of about 85% of their fertilizing ability. Although the inhibitory effect of different anti-sperm antibodies on fertility has been known for several years (Menge, 1970; Tzartos, 1979; Yanagimachi, Okada & Tung, 1981), antibodies to sperm-associated antigens of epididymal origin have only recently been shown to produce this effect in rabbits and hamsters (Moore, 1981). The absence of sperm agglutination and effects on motility in our experiments adds confidence to our results, eliminating this potential source of error in the work of Moore (1981).

Antiserum to protein DE reacts with the epididymal epithelium and with epididymal, but not testicular, spermatozoa (Kohane, Cameo, Piñeiro, Garberi & Blaquier, 1980a), suggesting that the antigen originated in the epididymis and was added to spermatozoa, rather than modifying a pre-existing sperm antigen during epididymal transit. Wong & Tsang (1982) have demonstrated the presence of high affinity receptors for an epididymal protein of 32 000 molecular weight (equivalent to our protein DE) on rat spermatozoa.

Our experimental approach cannot exclude a possible steric hindrance to gamete recognition, caused by the presence of immunoglobulins on the sperm surface. However, the data can also be interpreted as supporting the hypothesis proposed for the role of equivalent epididymal glycoproteins in the hamster (Cuasnicú *et al.*, 1984a, b; González Echeverría *et al.*, 1984). Based on the effect of an androgen-free preparation of epididymal glycoproteins upon immature hamster spermatozoa, we postulated that these moieties participate in the assembly or activation of a site for oocyte recognition on the sperm surface during epididymal maturation.

The ability of immunoglobulins to reach the epididymal lumen in sufficient numbers to block such maturation antigens is a key issue. The existence of a permeability barrier in the epididymal epithelium for substances of molecular weight >5000 is well documented (Cooper & Waites, 1973; Hinton & Howards, 1981; Wong *et al.*, 1983). However, Weininger *et al.* (1982) demonstrated that IgG penetrates the lumen of the rabbit epididymis *in vivo*, reaching a calculated ratio of 40 000 molecules IgG/spermatozoon. If a similar phenomenon occurred in the rat, the concentration of IgG would be enough to neutralize the estimated 400–600 molecules of rat epididymis 32K protein bound per spermatozoon (Wong & Tsang, 1982). However, Wong *et al.* (1983) report the absolute restriction to the entry of iodinated antibodies to rat 32K protein into the rat epididymal lumen *in vivo*. The result differs from our finding that rabbit antibodies to protein DE penetrate cultured rat epididymal tubules and decrease the fertility of contained spermatozoa almost to the same extent as does direct exposure to antiserum. However, the integrity of the specific permeability barrier in cultured epididymal tubules remains to be established.

This work was partly supported by NICHD grant 1 RO/HD 15920.

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Received 12 March 1984