Decreased Fertility and Motility of Spermatozoa from Rats Immunized with a Prealbumin Epididymal-specific Glycoprotein

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This study investigated the effects on the progressive motility, zona-binding capacity, and fertility of spermatozoa from the cauda epididymidis of adult male rats that were actively immunized against an acidic glycoprotein secreted by the epididymis. The percentage of motile spermatozoa was less than 5% in nine of ten rats that received the epididymal antigen, and 40 to 50% in eight of the 10 control rats. In animals immunized against the antigen, there was a dramatic decrease, but not a complete suppression, in the capacity of epididymal spermatozoa to bind the zona pellucida as compared with the non-immunized controls. Fertility was decreased two weeks after the end of the treatment, but partial restoration of fertility was observed 6 months later.

Key words: acidic, epididymal, glycoprotein, spermatozoa, rat, immunization, fertility.

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During the first steps of fertilization, spermatozoa must recognize the zona pellucida in order to bind to and penetrate ova. This capacity develops as spermatozoa pass through the epididymis, parallel to the development of fertilizing ability, and is associated with surface changes on the sperm head (rat: Orgebin-Crist and Fournier-Delpech, 1982; mouse: Saling, 1982). Several investigations have ascertained that molecular changes occur on the surface of spermatozoa after they leave the testis (Olson and Danzo, 1981; Voglmayr et al, 1983). Some of these modifications involve acidic proteins secreted by the epididymis that bind to the sperm surface and probably play a critical role in the acquisition of fertilizing ability (rabbit and hamster: Moore, 1981).

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In the rat, a glycosylated protein is secreted by the epididymis under the stimulation of testosterone. It has been identified by electrophoresis, followed by PAS staining, as epididymal-specific prealbumin 3 at *p*H 8.3 (Fournier-Delpech et al, 1973) or as protein D-E (Garberi et al, 1979). Binding sites for this protein have been localized on the membrane covering the acrosome and the sperm midpiece (Kohane et al, 1980; Bayard et al, 1981). These results suggest that a sperm antigen of epididymal origin may play a role in the membrane changes on spermatozoa that accompany the development of fertilizing capacity in the epididymis.

The objective of the present study was to examine the fertility of adult male rats actively immunized against an acidic epididymal glycoprotein. This glycoprotein was extracted using its affinity for Concanavalin A, and was fractionated by non-denaturing electrophoresis at *p*H 8.3 to obtain the prealbumin components.

Materials and Methods

Adult male rats of proven fertility and immature 26- to 28-day-old female rats of the Wistar 03 INRA strain were used. Gonadotropins were purchased from Intervet (Angers, France). The protein calibration kit was purchased from Sigma (St. Louis, MO).

Preparation of the Antigen

Twenty-five grams of cauda epididymidis from 100 male rats were recovered immediately after death and minced in 30 ml of cold saline, stirred at 2 C for 15 minutes, and then centrifuged for 20 minutes at 4 C. The

tissue and spermatozoa were discarded and the supernatant was applied to a Con A agarose column to extract glycoproteins having an affinity for Concanavalin A. After extensive washing with 0.1 M aceto-acetic buffer (0.1 M Na acetate in 0.1 M acetic acid, *p*H 5.6, containing 5 mM Ca++ and 5 mM Mg++), the glycoproteins were eluted with 1 M D-methylmannoside in the buffer, dialyzed against distilled water, and lyophylized. The lyophylate was dissolved in 10 mM Tris-Glycine buffer at *p*H 8.9, containing 20% sucrose, and applied to 7% acrylamide slab gels (14 × 10 × 0.2 cm) for electrophoresis (Davis, 1964). The prealbumin components were separated by electrophoresis, dialyzed against distilled water, and then lyophylized. The composition of the protein complex was assessed on SDS-polyacrylamide gels containing 15% acrylamide (Jones et al, 1980), and is referred to as rat epididymal-specific prealbumin.

Immunization Schedule

Thirty-three rats were divided into two groups: **Group 1 (20 rats):** The rats were assigned to two subgroups. Rats in the first subgroup (group 1A; n = 10) received 50 μg of rat epididymal-specific prealbumin in 50 μl saline emulsified with 50 μl of Freund's adjuvant in one injection/week for 4 weeks. The first injection was given intrasplenically using Freund's complete adjuvant. The next injections were made subcutaneously using Freund's incomplete adjuvant. The rats of the second subgroup (group 1B: controls; n = 10) were treated identically except that the antigen solution was replaced by 50 μl of protein-free saline. Three weeks after the last injection, the rats were killed. The presence of antibody against the epididymal glycoprotein was checked in the blood plasma by a precipitate at the interface between the serum and a solution of antigen in saline (1 mg/ml) in a capillary tube. Spermatozoa of the cauda epididymidis were recovered by micropuncture and immediately placed in Toyoda and Chang's (1974) medium either at 37 C for visual estimation of the percentage of progressively motile spermatozoa, or at 0 C to test the zona binding capacity as described below.

Group 2 (13 rats): Each rat selected had a fertility level of 100% prior to treatment. These rats were immunized with the antigen in Freund's adjuvant, the schedule of injections being the same as for group 1A. Two weeks after the last injection, each animal was tested to assess fertility. A second fertility test was performed 6 months later after the end of the treatment. The blood from this group of animals was not evaluated for the presence of an antibody against the epididymal antigen.

Zona Pellucida Binding Capacity of Distal Epididymal Spermatozoa

Zona binding capacity was measured as the capacity of spermatozoa to bind to the zonae of cumulus-free oocytes.

Preparation of Cumulus-Free Oocytes: Superovulation was induced in 26- to 28-day-old female rats by injecting PMSG (15 IU/250 μl intraperitoneally in the morning) and hCG (2.5 IU/250 μl intraperitoneally 53 hours later). Oocytes were recovered from the oviducts 15 to 20 hours after the hCG injection and placed in the medium of Toyoda and Chang (1974). Cumuli were dispersed by incubation for 15 minutes at room temperature in a medium containing hyaluronidase. The cumulus-free oocytes were then washed in the medium and stored at 0 C for 1 to 2 hours.

Preparation of Spermatozoa: Spermatozoa extracted from the cauda epididymidis were diluted in cold medium (1.5×10^6 and 8×10^6 spermatozoa/ml) and then stored at 0 C until assay. Storage time was always less than 1 hour.

Binding Assay: Ten eggs were added to 100 μl of the sperm suspension in a watch glass, and incubated for 1 hour at 0 C with gentle agitation every 10 minutes. At the end of the incubation, the eggs were recovered, washed three times in cold medium, and mounted for observation under a phase contrast microscope to assess the number of spermatozoa bound per egg.

Fertility Assay

Each male of group 2 was placed with four females of proven fertility for 1 week. Fertility was calculated as n/4 × 100, where n is the number of females giving birth.

Statistical Analysis

Results were analyzed by chi square.

Results

Characteristics of the Antigen

The antigen was the most acidic glycoprotein that can be eluted from insolubilized Con A with D-methylmannoside. It migrates in the prealbumin area when electrophoresis is performed on polyacrylamide gels under non-denaturing conditions (Fig. 1). When applied to SDS-polyacrylamide gels for electrophoresis after elution from the non-denaturing gel, it appears to consist of two major proteins having molecular weights of 18,500 D and 32,000 D, respectively (Fig. 2).

Immunization of the Rats

One month after the last injection of the glycoprotein antigen, antibodies were present in the blood of nine of 10 rats from group 1A, whereas no antibodies were detected in any of the control rats (group 1B).

Motility and Zona Binding Capacity of Epididymal Distal Spermatozoa

The percentage of progressively motile spermatozoa was less than 5% in nine of the 10 rats of group 1A that received the epididymal antigen, and 40 to 50% in the tenth rat. This animal was one of the nine with antibodies present in the blood. By contrast, the percentage of progressively motile spermatozoa was 40 to 50% in eight of the control rats and 15 to 20% in the remaining two. The capacity of spermatozoa from the cauda epididymidis of animals immunized

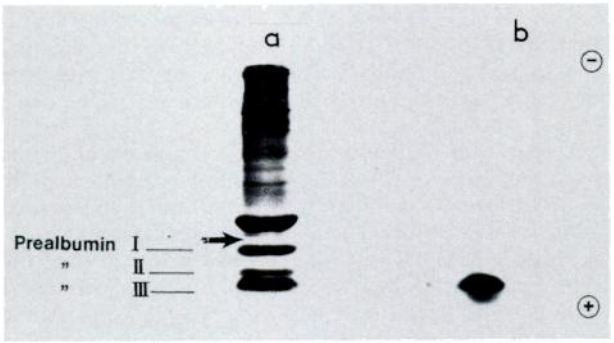

Fig. 1. Coomassie blue staining of plasma proteins from the cauda epididymidis (a) and rat prealbumin epididymal-specific glycoprotein extract (b) on non-denaturing polyacrylamide gel. The arrow indicates the location of albumin.

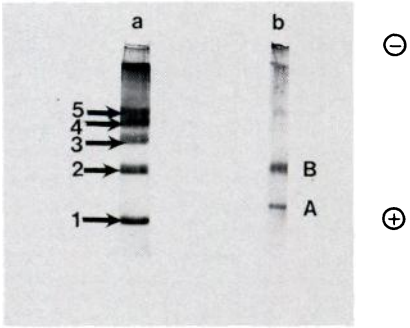

Fig. 2. Coomassie blue staining of calibration proteins (a: 1 = 14,000 D; 2 = 32,000 D; 3 = 48,000 D; 4 = 64,000 D; 5 = 68,000 D) and rat prealbumin epididymal-specific glycoprotein (b: A = 18,500 D; B = 32,000 D) on SDS-polyacrylamide gel.

against the antigen was significantly reduced, but not completely suppressed, when compared with spermatozoa from the non-immunized controls.

Fertility

The fertility of the immunized rats of group 1B was reduced 2 weeks after the end of treatment, and in some males was completely lost. A small improvement, but not a complete recovery, of fertility was observed when the animals were assessed 6 months after the end of treatment (Table 2).

Discussion

The present study indicates that immunization of adult male rats against an epididymal prealbumin glycoprotein reduces their fertility.

The antigen injected is the most acidic of the epididymal proteins, migrating at the anode at *p*H 8.3 during electrophoresis on polyacrylamide gels under non-denaturing conditions (Fig. 1); it corresponds to the specific acidic glycoprotein secreted by the epididymis under the control of testosterone and adsorbed on the surface of spermatozoa from the distal portion of the epididymis (Lea et al, 1978; Bayard et al, 1981).

On SDS gel electrophoresis, rat epididymal-specific prealbumin appears to be composed of two major proteins with molecular weights of 32,000 D and 18,500 D, respectively. The 32,000 D component probably corresponds to the 32,000 D protein that binds to maturing spermatozoa and that was characterized by Lea et al (1978). The 18,500 D protein strikingly resembles the 18.5 K_d protein (Jones et al, 1980) secreted *in vitro* by the epididymis under the control of testosterone, and which is detected on the flagella of mature epididymal spermatozoa (Jones et al, 1982). The binding of epididymal secretory proteins on spermatozoa has been clearly shown in the rat (Brooks and Tiver, 1983). This could explain why, in our study, immunization with epididymal fluid preparations causes reduced fertility.

In our experiments, the decline in the zona binding capacity of spermatozoa from the cauda epididymidis (Tables 1 and 2) could have resulted from the immunization of these rats against rat prealbumin epididy-

TABLE 1. Zona Pellucida Binding Capacity of Spermatozoa of the Cauda Epididymis after Immunization Against Prealbumin Epididymal-specific Glycoprotein

Treatment	Number of Spermatozoa Inseminated *in vitro*/100 μl	Number of Eggs Observed	Number of Eggs Having n Spermatozoa Bound (%)		
			n = 0	n = 1-10	n = 10
Control					
1B (Freund's adjuvant + saline)	0.15×10^6	124	(10 ± 10)*	20 ± 20	70 ± 13
Immunized					
1A (Freund's adjuvant + prealbumin epididymal-specific glycoprotein)	0.15×10^6	118	(55 ± 35)†	45 ± 40	0
	0.8×10^6	39	(25 ± 15)	60 ± 15	10 ± 10

*mean ± SEM/rat.
†Statistically different from control $P < 0.05$.

TABLE 2. Effect of Immunization Against Prealbumin Epididymal-specific Glycoprotein on the Subsequent Fertility of Rats after the End of Treatment

Treatment n = 13	Delay Between the Last Injection and the Fertility Test	Percentage of Males Having Fertility of:				
		100%	75%	50%	25%	0%
Control*		100	0	0	0	0
Immunized	2 weeks	0	0	25	45	30
	6 months	0	15	55	30	0

*Controls = animals before the beginning of the treatment.
At the various levels of fertility (F = 75, 50, 25, or 0%), the differences between the two groups of immunized animals (2 weeks or six months) were statistically significant ($P < 0.05$).

mal-specific glycoprotein, which is a mixture of 32,000 D and 18,500 D moieties. The respective roles of these components, however, have not been investigated in this study. Antibodies were observed in the blood of nine of the 10 rats treated with this epididymal-specific glycoprotein, as contrasted with none of the control rats, which were treated with Freund's adjuvant without antigen. Androgen-dependent epididymal secretory proteins, such as rat prealbumin epididymal-specific glycoprotein, have been shown to be involved in the development of zona binding ability by spermatozoa during epididymal transit (Orgebin-Crist and Fournier-Delpech, 1982; Cuaniscu et al, 1984). The partial restoration of fertility (Table 2) 5 months after the end of the treatment could be due to a decline in the induced immunity.

In other species, immunization with sperm membrane antigen or epididymal glycoproteins has been shown to reduce fertility. In the rabbit, artificial insemination with spermatozoa treated with an antiserum against the plasma membrane resulted in decreased fertility, due to the inability of spermatozoa to penetrate the zona pellucida (O'Rand, 1981). In this case, the autoantigen was of testicular origin. In rabbit, fertilizing ability declined when cauda epididymal spermatozoa were preincubated with univalent immunoglobulin fragments against various epididymal glycoproteins before insemination (Moore, 1981). In addition, the decreased fertility of female rats immunized with spermatozoal proteins (Mettler et al, 1983) indicates that anti-sperm antibodies are also effective *in vivo* in the female tract.

How immunization alters epididymal spermatozoa remains to be elucidated. These changes could result from the presence of a class of immunoglobulins in the lumen that combines with the epididymal glycoproteins and prevents their binding to the sperm surface. This tentative explanation is consistent with the work of Weininger et al (1982), who reported that specific immunoglobulins were present in the epididymal fluid of rabbits following systemic immunization with dinitrophenylated bovine gamma globulin. Wong et al (1983), however, have reported that a blood-epididymal barrier restricts the passage of immunoglobulins from the blood into the rete testis fluid and then into the epididymis in rats passively immunized against rat epididymal proteins. Another explanation for the declined fertility could be that the immunity process changes the secretory function of the epithelial cells at the site of epididymal-specific glycoprotein synthesis and alters the plasma within the epididymis (Lea et al, 1978; Bayard et al, 1981). Further immunologic and histologic data must be obtained to elucidate the mechanism of action by which immunization with a prealbumin epididymal-specific glycoprotein affects male fertility.

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International Meeting on Hormonal Therapy of Prostatic Diseases: Basic and Clinical Aspects

An international Symposium on "Hormonal Therapy of Prostatic Diseases: Basic and Clinical Aspects" will be held at the Michelangelo Hotel (Milano, Italy) from May 21 to May 24, 1986. The meeting will be planned by an International Scientific Committee formed by: Bartke A. (USA), Bruchowsky N. (Canada), Geller J. (USA), Motta M. (Italy), Robinson J. (U.K.), Serio M. (Italy), Voigt K.D. (Germany). The program will include invited lecturers as well as sessions of free communications and/or poster presentations on the following topics: The normal prostate: morphological, biochemical and hormonal aspects; The pathological prostate (BPH, Carcinoma, etc.): morphological, biochemical and hormonal aspects; Therapeutic approaches in prostatic diseases (animal and human studies). For further information regarding the program, please contact the Scientific Secretaries. For registration, travel and logistic information, please contact the Organizing Secretariat.

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