Inhibition of the Cumulus Dispersing and Hyaluronidase Activities of Sperm by Heterologous and Isologous Antisperm Antibodies[1] (36551)

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The cumulus mass surrounding the egg is one of the final physical barriers to sperm–egg interaction in most mammals. Sperm penetration of this barrier is mediated by a sperm-borne enzyme, hyaluronidase (1, 2). In view of this apparently essential role in the reproductive process it seemed of interest to determine if rabbit and human sperm hyaluronidases, like bovine testicular hyaluronidase (3), are vulnerable to immunological inactivation and if the cumulus dispersing action (CDA) of sperm and hyaluronidase is prevented by appropriate antibodies of heterologous and especially isologous origin. This proved to be the case. The results are consistent with the suggestion (4) that at least some instances of apparent immunologically induced infertility in women result from antibodies to sperm hyaluronidase and provide a more rational approach to fertility control by immunization with seminal components. Finally, this is the first case of a reasonably well-known mammalian sperm substance with recognized function in reproduction that is inhibited by antibodies of heterologous and isologous origin. Part of this study was reported in abstract form (5).

Materials and Methods. Guinea pigs, goats and virgin female rabbits were immunized by injection of rabbit epididymal sperm, ejaculated semen or seminal plasma from vasectomized males in Freund's adjuvant emulsion (6, 7). Antihuman semen antibodies were similarly prepared in rabbits. Control (preimmune bleeding) and immune sera were heated (56°, 30 min) to destroy nonspecific hyaluronidase inhibiting activity (8).

Univalent (Fab) antibody fragments were used in most of the experiments, including those of Fig. 1, and Tables I, II and III to avoid secondary physical effects (*e.g.*, agglutination, precipitation) by the antibody. These were prepared by papain digestion (6, 9). Most papain digests of immune and control γ-globulin alone dispersed the rabbit cumulus even after iodoacetamide alkylation and dialysis against the phosphate buffered saline (PSB, *e.g.*, 0.01 *M* PO_4, 0.85% NaCl, pH 7) in which most experiments were performed. This nonspecific cumulus dispersing action did not occur after passage of the preparations through Sephadex G-25. Such chromatographed antibody was used in most of the experiments. Presence of univalent antibody was confirmed by Coombs (antiglobulin) sperm agglutination tests. Cumulus masses were obtained by flushing the oviducts of female rabbits 13 hr after intravenous administration of 75 IU of HCG (Mann Research Laboratories). Rabbit semen was collected by artificial vagina, epididymal (rabbit, guinea pig, mouse and rat) sperm from cauda epididymis and capacitated sperm by recovery of rabbit epididymal sperm from rabbit uteri 8–10 hr after intrauterine insemination and HCG (75 IU) injection. Capacitation was confirmed by recovery of cleaving eggs from test females inseminated intratubally 13–15 hr after HCG (75 IU) injection.

To test for inhibition of CDA, 0.03 ml of a 25×10^6 sperm/ml suspension or sperm extract was first incubated in 0.09 ml of buffer, control or immune globulin solutions for 15 min at 37°. Subsequently, 0.09 ml (approx. 5.6×10^5 sperms) of the treated suspension was mixed in a depression slide with 0.06 ml buffer containing one rabbit cumulus mass. Progress of cumulus disper-

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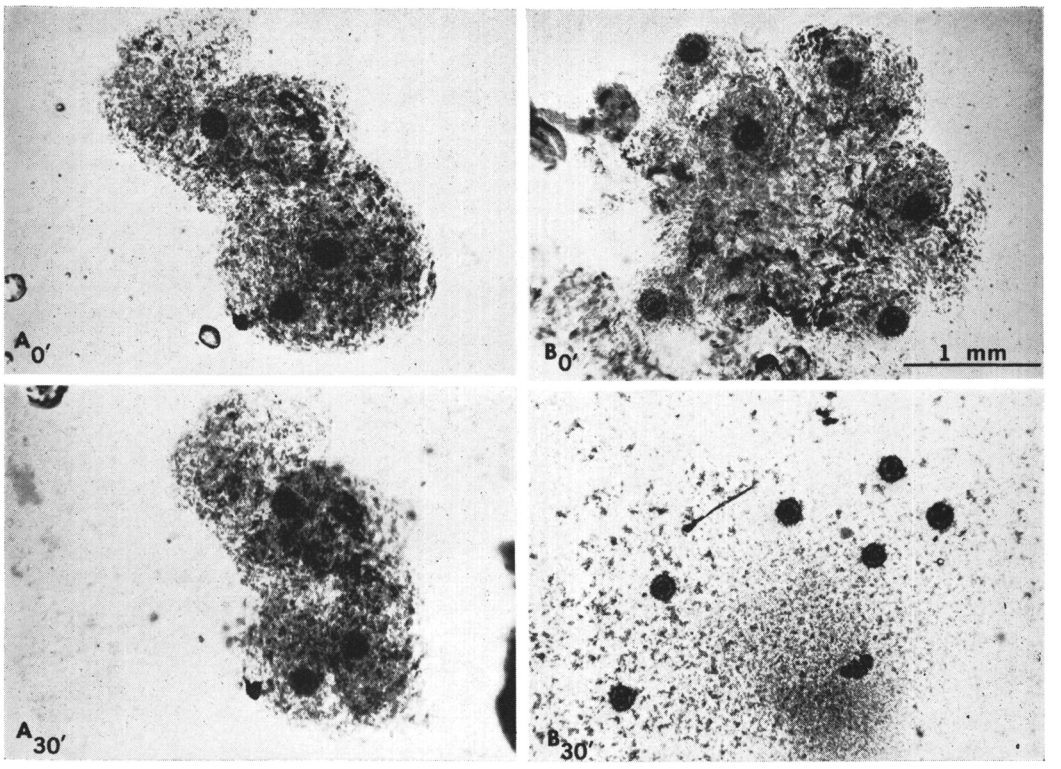

FIG. 1. Inhibition of rabbit sperm cumulus dispersing action by univalent antibody. (A_0') Rabbit cumulus containing three eggs and numerous follicle cells. Photo taken at time rabbit semen + papain digested goat antirabbit semen antibody mixture was added to cumulus. Discrepancy between sizes of cumulus masses [e.g., (A_0') vs (B_0')] results from individual cumulus mass variation. The smaller mass (e.g., less material to depolymerize) was selected for the experimental treatment (inhibition by antibody) to provide conservative bias. (A_{30}') Same field of view as A_0' but 30 min later. Cumulus is intact; eggs and follicle cells are still entrapped in the cumulus matrix. (B_0') Rabbit cumulus mass containing six rabbit eggs and many small follicle cells. Photo taken at time (0 min) rabbit semen + papain digested control goat globulin mixture was added to cumulus. (B_{30}') Same field of view as B_0' but photographed 30 min later. The cumulus mass has dispersed, the six eggs and the follicle cells are free and resting on the bottom of the slide, e.g., positive cumulus dispersion by seminal hyaluronidase.

sion was followed microscopically for 30 to 60 min. Controls in all experiments included cumulus masses in buffer, antibody and preimmunization globulin without semen. Cumulus failed to disperse in these preparations except in the case of papain digests prior to Sephadex G-25 filtration (see above).

The antibody preparations were also examined quantitatively for inhibition of hyaluronidase activity of sperm extracts in a turbidimetric procedure modified from Tolksdorf *et al.* (10). Antibody and sperm extracts were incubated (37°, 15 to 30 min), subsequently mixed with hyaluronic acid (pH 5.4) and again incubated (37°, 30 min). Acidified (pH 4.0) albumin with or without $FeCl_2$ (5×10^{-4} *M*) was then added to terminate hydrolysis and develop turbidity. Controls included hyaluronic acid and globulin alone, sperm extract and globulin without hyaluronic acid and finally hyaluronic acid and buffer alone. The turbidity was read with Klett-Summerson colorimeter and filter No. 42. Protein concentrations were determined by the biuret (11) and Lowry *et al.* (12) methods using cystalline bovine serum albumin as standard.

Results. Rabbit semen pretreated with con-

TABLE I. Inhibition of CDA and Hyaluronidase Activities by Univalent Goat Antirabbit Semen Antibodies.[a]

Sperm or hyaluronidase	Rabbit			Commercial bovine testicular hyaluronidase	Epididymal sperm of		
	Epididymal	Ejaculated	Capacitated		Guinea	Mouse	Rat
CDA							
Goat antirabbit semen globulin	—	—	—	+	+	+	+
Control goat globulin	+	+	+	+	+	+	+
Hyaluronidase TRU inhibited/mg goat globulin							
Goat immune	4.35	>1.00	>0.34	0.00			
Goat control	0.66	0.00	0.00	0.00			

[a] + = Positive cumulus dispersing action (CDA) in 15 min and complete dispersion in 30 min, (e.g., Fig. $1B_{30}'$); — = No cumulus dispersion after 30 min exposure (e.g., Fig. $1A_{30}'$). TRU inhibited = turbidity reducing units of hyaluronidase activity inhibited (9). 0.00 = no inhibitions; >1 = complete inhibition of activity in the preparation, e.g., inhibitory activity in excess of value given. The reaction mixtures contained frozen–thawed sperm extract of hyaluronidase activity in the range of 75 to 820 TRU/ml, and 0.4–4.0 mg γ-globulin. This mixture was incubated for 15 min, and then added to 0.5 ml (0.2 mg) hyaluronic acid, final volume 1 ml.

trol γ-globulin or buffer completely dispersed the rabbit cumulus in 30 min freeing the eggs and cumulus cells (Fig. 1B). However, semen pretreated with antibody had no visible effects on the cumulus (Fig. 1A). The eggs and cumulus cells remained embedded in the intact cumulus mass. The CDA of epididymal, ejaculated and capacitated rabbit sperm suspensions was inhibited by both goat (Table I) and guinea pig antirabbit sperm antibodies. Antibodies to rabbit epididymal sperm (guinea pig origin) and to rabbit ejaculated whole semen (guinea pig and goat origin) were both effective in either the native or univalent (Fab) forms.

The inhibition of CDA by antirabbit semen antibody was highly specific (Table I). The rabbit cumulus was dispersed by bovine hyaluronidase, and guinea pig, mouse and rat sperm suspensions equally in the presence or absence of antirabbit semen antibody. This species specificity of inhibiting action implies that the antibody acts on a sperm antigen(s) and not on the cumulus. This was confirmed by washing experiments, *e.g.*, cumulus masses washed from antirabbit semen antibody were rapidly dispersed by rabbit semen. Rabbit sperm washed from the antibody did not disperse the cumulus. Antibodies prepared in guinea pigs against seminal plasma from vasectomized male rabbits failed to inhibit CDA of rabbit sperm. This is consistent with the fact that sterile semen from vasectomized males lacks CDA and hyaluronidase activity (13).

In the quantitative tests (Table I), antibodies (both native and Fab fragments) to rabbit semen or epididymal sperm strongly inhibited the hyaluronidase activity of rabbit sperm extracts but not hyaluronidase of bovine origin.

A question of some interest is whether antibody treatment results in inactivation of only a part (*e.g.*, "surface layer") of the hyaluronidase of an individual spermatozoan or of the cell's entire complement of hyaluronidase. To examine this, suspensions of washed sperm were mixed with antibody preparations, subsequently washed in saline to remove excess antibody and finally extracted for hyaluronidase by freeze–thawing. As shown in Table II, extracts of the antibody treated sperm had only about 50% of the hyaluronidase activity found in compara-

TABLE II. Recovery of Hyaluronidase Activity in Frozen–Thawed Extracts of Antibody Treated Rabbit Sperm.[a]

Expt. no.		Immune[b] (in PO_4 buffer)	Globulin[b] control (in PO_4 buffer)	PO_4 buffer[b] control	% Inhibition[c]
Native globulin					
1	Extract alone	28.5	86.0	65.2	62
	Extract + washes	33.3	92.2	89.7	63
2	Extract alone	45.0	78.5	89.1	46
	Extract + washes	49.3	150.6	134.0	66
Papain digested globulin					
3	Extract alone	38.6	135.8	79.6	64
	Extract + washes	38.6	162.7	87.3	69
4	Extract alone	122.0	190.4	197.5	37
	Extract + washes	131.4	211.6	220.1	39

[a] Samples of washed, ejaculated rabbit sperm were mixed with aliquots of goat antirabbit semen globulin, control goat globulin and PBS. All mixtures contained approximately 5×10^7 sperm. Mixtures in Expts. 1 and 2 contained 140 mg globulin in a final volume of 2.55 ml; in Expts. 3 and 4, 60 mg of univalent globulin in approximately 3.5 ml. After incubation (37°, 15 min), samples were centrifuged and washed twice in saline. Sperm were then diluted to 0.5 ml with PBS and extracted by 10 freeze–thawing cycles.

[b] Values are TRU/10^8 sperms; the upper values in each experiment are from extracts of washed sperm, lower values are total activities of extracts plus washes, both calculated as TRU/10^8 sperm from the original treated sample.

[c] Calculated from activities using average of control globulin and buffer control as 100% activity.

ble extracts of control globulin treated sperm suspensions.

The first wash from the antibody treated sperm in all experiments except no. 3 possessed some hyaluronidase activity even though these washes all agglutinated untreated rabbit sperm [direct agglutination in Expts. 1 and 2; Coombs (antiglobulin) agglutination in Expts. 3 and 4]. This implies that antihyaluronidase antibody excess was achieved only in Expt. 3.

Female rabbits can be rendered infertile by isoimmunization with rabbit semen (7, 14). Accordingly, virgin female rabbits were isoimmunized with rabbit sperm and the resulting antibodies were examined for inhibition of rabbit sperm CDA and hyaluronidase activity (Table III). The post immunization γ-globulins from all five rabbits inhibited rabbit sperm CDA and rabbit sperm hyaluronidase activity. Although the antihyaluronidase activities varied considerably, the variation was not related to the source (epididymal vs ejaculated) of immunizing sperm. The two strongest preparations (rabbits nos. 2 and 4, Table III) were examined for inhibition of epididymal and capacitated sperm hyaluronidases in addition to ejaculated sperm hyaluronidase. The lower inhibitory activity for capacitated sperm hyaluronidase suggests contaminating hyaluronidase of other immunological specificity (possibly from uterine leukocytes) or antibody neutralization by inactive enzyme.

Human sperm contains hyaluronidase (15). Rabbit antihuman semen antibodies were examined for inhibition of rabbit cumulus dispersion and hyaluronidase activity by human sperm hyaluronidase in preliminary experiments. Human sperm extracts did disperse rabbit cumulus in the presence of buffer or control γ-globulin, but failed to do

TABLE III. Inhibition of Rabbit Sperm CDA and Hyaluronidase Activity by Univalent Antisperm Isoantibodies from Virgin Female Rabbits.[a]

	Immunized with rabbit semen				Immunized with epididymal sperm				
Virgin rabbit no.:	1		2		3		4		5
Source of test sperm and hyaluronidase	Ejac.	Epid.	Ejac.	Capac.	Ejac.	Epid.	Ejac.	Capac.	Ejac.
CDA									
Immune globulin	—[b]	—	—	—	—	—	—	—	—
Control globulin	+	+	+	+	+	+	+	+	+
Hyaluronidase TRU inhibited/mg rabbit globulin									
Immune globulin	0.37[b]	>10.0	>10.0	1.84	1.13	3.92	3.65	0.65	0.54
Control globulin	0.00	0.00	0.00	0.00	0.00	0.20	0.00	0.00	0.00

[a] See Legend to Table I for symbols. Frozen–thawed sperm extract hyaluronidase activities were in the range 75–820 TRU/ml; the papain digested globulin (0.3–1.0 mg/ml) preparations were pooled from five bleedings for each rabbit. All globulin samples except the rabbit no. 5 aliquot used with epididymal sperm extract were heated (56°; 30 min) to destroy nonspecific hyaluronidase inhibitor (6). Globulins from all five rabbits were tested on ejaculated sperm for CDA inhibition and extracts of ejaculated sperm for hyaluronidase inhibition. The same extract of ejaculated sperm (312 TRU/ml) was used for hyaluronidase inhibition with globulin from rabbits 2, 3 and 4. Globulins from rabbits nos. 2 and 4 were tested on epididymal and capacitated sperms and sperm extracts in addition to ejaculated sperm preparations.

[b] The papain digested pooled globulin from the five bleedings of rabbit no. 1 did not inhibit CDA in the standard 30 min test. Serum (undigested) from a subsequent bleeding did meet this test. The hyaluronidase inhibition activity value is for papain digest of the pooled bleedings.

so in presence of antihuman semen antibody. Both native and univalent rabbit antihuman semen antibodies were effective. The antihuman semen antibody did not inhibit the CDA of rabbit semen, again indicating specificity. Evidently, human sperm hyaluronidase is antigenic in rabbits.

Discussion. It seems clear from the effects of univalent antibody in this study that the enzymatic active site or a neighboring region of rabbit and human sperm hyaluronidases are antigenic and that the antibodies inhibit the enzyme either by direct combination with the active site or by steric effects, not by secondary physical effects (*e.g.*, precipitation of hyaluronidase). In addition, antibodies to rabbit sperm inhibit hyaluronidase activity with a specificity comparable to other antihyaluronidase antibodies, *e.g.*, antibovine (3, 4), antibacterial hyaluronidases (16) and antihyaluronidases to other sperms (17). It is evident that antihyaluronidase antibodies are effective inhibitors of the CDA of sperm. Like other hyaluronidase inhibitors (1) antihyaluronidase antibodies can prevent passage of sperm through the cumulus. This was confirmed by direct observation with phase optics. Although antisperm antibodies can inhibit reproduction by action elsewhere in the female tract (6), the inhibition of CDA suggests that the antihyaluronidase antibodies may block fertilization by preventing sperm penetration of the cumulus. This view is consistent with the fact that only antibodies to sperm (epididymal or ejaculated) inhibit CDA, hyaluronidase and conception (18). Antibodies to seminal plasma alone have none of these effects. Confirmation of this view will require an immunologically pure antigen–antibody system.

Extraction of antibody pretreated, washed sperm yielded only about 50% of the hyaluronidase activity of controls. This is interpreted to mean that at least half the hyaluronidase of intact sperm is physiologically accessible to the antibody. Other explanations are possible, *e.g.*, differential death and release of hyaluronidase from the antibody

treated samples followed by inactivation of the hyaluronidase in solution. As yet there is no evidence for this and the possibility seems especially unlikely in the case of univalent antibody treated samples (6).

The isoantibody preparations with antihyaluronidase and anti-CDA activities (Table III) were obtained using an immunization protocol employed by Menge (7) to produce female infertility in rabbits. In view of these findings, and the demonstration of human sperm hyaluronidase antigenicity in rabbits, it would seem important to examine sera from vasectomized men and infertile women, and especially sera with sperm agglutinating activity indicative of antisperm antibodies, for inhibition of human sperm hyaluronidase and CDA.

Finally, it should be noted that others (4, 19, 20) observed CDA inhibition by antisperm antibodies and some (4) appreciated the potential significance of the results. However, the necessary follow-up experiments were not performed and interest lapsed, probably from misunderstandings about specificity (20) and uncertainty of mechanism of antibody inhibition (21).

Summary. Rabbit semen, but not rabbit seminal plasma (vasectomized rabbits), rapidly disperses the cumulus mass surrounding newly ovulated rabbit eggs. Following exposure to antisemen antibodies (goat or guinea pig origin) rabbit semen and sperm extracts fail to disperse the cumulus. Likewise, rabbit sperm extracts failed to depolymerize hyaluronic acid in the presence of antisemen antibody. These inhibitory effects are produced by both bivalent antibody and by univalent (Fab) antibody fragments with a high order of species specificity. Antiepididymal sperm antibodies, but not antiseminal plasma antibodies also have the inhibitory properties. Approximately 50% of the extractable hyaluronidase activity of ejaculated rabbit sperm is vulnerable to antibody inhibition prior to extraction. Isoimmunization of virgin female rabbits with rabbit semen results in isoantibodies that inhibit rabbit sperm cumulus dispersing action and hyaluronidase activities of epididymal, ejaculated and capacitated rabbit sperm extracts. Human semen hyaluronidase disperses rabbit cumulus and depolymerizes hyaluronic acid. Both of these effects are inhibited by rabbit antihuman semen antibodies.

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