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Reduction of fertility in female rabbits and mice actively immunized with a germ cell antigen (GA-1) from the rabbit

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Female rabbits and mice were actively immunized against germ cell antigen (GA-1) of 63 kDa molecular mass isolated from rabbit sperm and testis. There was a significant ($P < 0.05$) reduction of fertility in rabbits actively immunized with GA-1 as compared to controls, as seen by the percentage of 9-day implants/corpora lutea ratio (GA-1, 36.3%; controls, 85.7%). In mice, there was again a significant ($P < 0.01$) reduction in fertility as seen by mean 7–9 day implants ± S.D. per mated mouse actively immunized with GA-1 whether through the intraperitoneal route (GA-1, 1.2 ± 1.6; controls, 8.0 ± 3.4) or through the subcutaneous/intramuscular route (GA-1, 3.8 ± 3.4; controls, 10.1 ± 3.9). The antisera from these actively immunized animals were negative for sperm agglutinating and immobilizing antibodies. In the Western blot enzyme-immunobinding procedure, the antisera showed specific binding to a single protein of 63 kDa. The incidence of fertilization of eggs recovered from rabbits inseminated with anti-GA-1 antibodies-treated sperm was not significantly different from control rabbits. The percentage of fertilized eggs obtained from rabbits inseminated with anti-GA-1 antibodies-treated sperm that reached the blastocyst stage upon in vitro incubation, however, was significantly less than that for embryos obtained from rabbits inseminated with control serum-treated sperm. Incubation of normal fertilized eggs in vitro with the antibodies did not affect development. Neither antiserum nor immune uterine fluid reacted with 4-day blastocysts in the indirect immunofluorescence technique. It is concluded that active immunization with GA-1 results in post-fertilization reduction of fertility in rabbits and mice by inhibiting early embryonic development.

Key words: *fertility regulation, sperm antigen, immunoreproduction, Western blot*

Introduction

It is more than three quarters of a century ago that the first documented attempts to immunize with male gamete antigens were initiated. In 1899, two immunologists, Landsteiner and Metchnikoff independently demonstrated that injection of sperm

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or testicular homogenate from heterospecies produced sperm-reacting antibodies in animals. Since then it has been amply documented that deliberate immunization of female animals of numerous species with preparations of isologous sperm or mature testis results in infertility due to either fertilization failure or preimplantation embryo mortality or to both (reviewed by Jones, 1982).

However, use of whole sperm or testicular homogenate is not desirable because of the presence of some antigens on germ cells which may be shared with other somatic tissues (Freund et al., 1955; Mathur et al., 1981; Jorgensen and Moller, 1983). A few sperm-specific antigens have been isolated and characterized. Some of these antigens are involved in immunoregulation of fertility. Immunization with germ-cell-specific lactate dehydrogenase (LDH-C_4) has been shown to reduce fertility in mice, rabbits and baboons (Goldberg et al., 1981). Antiserum to rabbit sperm autoantigen (RSA-I) inhibited fertility in female rabbits (O'Rand, 1981). There is a need to isolate and characterize additional specific antigens which are involved in fertilization processes and which can elicit an immune response sufficient to intercept fertility. It has been possible to identify and isolate such antigens by using hybridoma technology and immunoaffinity chromatography. There are reports of studies on monoclonal antibodies (MCAs) against sperm of mice (Feuchter et al., 1981; Schmell et al., 1982), rats (Gaunt et al., 1983), guinea pigs (Myles et al., 1981) and humans (Lee et al., 1982; Isahakia and Alexander, 1984). Recently, Saling and coworkers (Saling and Lakoski, 1985; Saling et al., 1985) described a series of MCAs against mouse sperm that blocked fertilization. Two of the antibodies prevented sperm penetration through the zona pellucida and another two MCAs inhibited fusion of the sperm plasma membrane with the plasma membrane of the egg.

We reported five germ-cell-specific monoclonal antibodies against rabbit sperm (7C6.3, 8C4.1, 8F4.3, 8F4.5, and 8C10.5), one of which (8C10.5) resulted in significant inhibition of fertility (Naz et al., 1983). By using this monoclonal antibody, we isolated and partially characterized an antigen of 63 kDa from rabbit germ cells (GA-1) (Naz et al., 1984b). The present study describes the effect of active immunization with purified GA-1 on the fertility of female rabbits and mice, and the effect of antibodies to GA-1 on fertilization and postfertilization embryo development in rabbits.

Materials and Methods

Rabbits used for the present study were of the random-bred New Zealand white strain. Semen was collected by the aid of an artificial vagina. Semen of good quality collected from three or more fertile bucks was pooled and used in artificial insemination. The 63 kDa antigen (GA-1) was isolated and characterized from rabbit sperm and testes by using monoclonal antibody (8C10.5) as described earlier (Naz et al., 1984b). Electrophoresis was performed on 10% polyacrylamide–SDS slab gels using the method of Laemmli (1970). Two other antigens of approximately 75 and 100 kDa (polymer upon reduction gave two bands of 58 and 28 kDa) were

isolated from rabbit germ cells by using immunoaffinity chromatography involving monoclonal antibodies 7C6.3 and 8F/G respectively. Both of these antibodies demonstrated a lack of effect on fertility. The antigens were used as controls for immunization of female mice.

Immunization

Sexually mature virgin female rabbits were immunized systemically at multiple subcutaneous and intramuscular sites against (a) GA-1 (in Tris-HCl buffer containing 0.6% sodium deoxycholate, pH 8.0), (b) detergent (0.6% sodium deoxycholate) solubilized rabbit testis (DOC-RT), an extract from which GA-1 has been purified or (c) buffer-DOC solution only. The first injection consisted of 0.2–0.3 ml of detergent preparation containing 150–250 μg of protein emulsified with an equal volume of Freund's complete adjuvant (FCA). Starting 1 wk after the primary injection, the rabbits were injected weekly for 3 wk with 75–100 μg of the antigen mixed with incomplete Freund's adjuvant (IFA). Two weeks after the last injection, the animals were bled through the midear artery and antiserum was collected and stored at $-20\,^{\circ}$C until use. Control rabbits were injected on the same schedule and volumes but without any protein antigen.

Three immunized rabbits were examined for development of local immunity in the genital tract after systemic immunization. Two rabbits were immunized against GA-1 and one against DOC-RT. They were anesthetized with sodium pentobarbital and the uterine horns after exposure through an abdominal midline incision, were ligated at the tubal and cervical ends with 2–0 nylon suture. Approximately 0.2–0.3 ml of the corresponding antigen (100 μg of GA-1 or DOC-RT) and 0.1 mg each of polyadenylic acid and polyuridylic acid (PAPU) in phosphate-buffered saline (PBS, pH 7.4) were injected into each uterine horn. Ten days after uterine ligation and challenge, the accumulated fluid was aspirated, concentrated by dialysis against dry sucrose and finally dialysed against multiple changes of PBS for 48 h.

CD-1 virgin female mice were immunized systemically against the different antigen preparations: GA-1, 7C6.3 Ag, 8F/G Ag, DOC-RT, and adjuvant control. The first injection was comprised of 0.1 ml of antigen preparation (50–75 μg) emulsified with an equal volume of FCA followed weekly for 3 wk with 25 μg of the antigen mixed with IFA. Four experiments were run; mice in trials 1 and 2 were immunized via the i.p. route and mice in trials 3 and 4 via s.c. and i.m. routes. One to two weeks after the last injection, mice were bled by retro-orbital puncture, antiserum was collected and stored at $-20\,^{\circ}$C until further use.

Control sera were collected from animals before immunization and from animals receiving systemic injections of Freund's adjuvant and the buffer-DOC mixture. Control uterine fluids were collected from rabbits receiving only PAPU. Both sera and uterine fluids were heated at 56 °C for 30 min before use.

Analysis of sera

The presence and titers of antibodies in the sera or uterine fluids were detected by the enzyme-linked immunosorbent assay (ELISA) using GA-1 as the antigen

coated on plates as described earlier (Naz et al., 1983), the gelatin agglutination technique (Kibrick et al., 1952), the sperm immobilization technique (Isojima et al., 1968), indirect immunofluorescence technique (Menge and Peegel, 1980) and Western blot enzyme immunobinding procedure (Naz et al., 1985). Antisera with ELISA titers of ≤ 1600 were examined for their effect on in vitro development of early embryos and in artificial insemination of rabbits. Sperm were treated with antisera (37°C, 30 min) to investigate their effect on fertilization and postfertilization embryonic development. The animals were artificially inseminated with 10^7 motile sperm in a final volume of 250 μl consisting of 200 μl of antiserum and 50 μl of semen appropriately diluted in Hank's balanced salt solution. Organ specificity of antisera was checked by absorption with spleen cells, washed ejaculated sperm, testicular homogenate, brain, kidney and liver powder (1 : 1, packed cell vol.) at 37°C for 1 h, followed by overnight incubation at 4°C as described earlier (Naz et al., 1984b). The indirect immunofluorescence technique was used to study the immunological cross-reaction of the antibodies with oocytes, two-cell embryos and blastocysts obtained 4 days postinsemination. Superovulation and blastocyst recovery followed procedures described previously (Menge and Peegel, 1980).

Fertility trials

One week after the last injection, rabbits were inseminated with 10^7 sperm suspended in 0.25 ml of Hanks' balanced salt solution and injected intravenously (i.v.) with 100 I.U. human chorionic gonadotropin. At 9 days postinsemination, the animals were anesthetized with sodium pentobarbital and the uterine horns were exposed through an abdominal midline incision. Fertility was determined by comparing the number of viable-appearing implantation sites in both uterine horns per number of corpora lutea in the ovaries.

In mice, fertility was tested by mating of immunized females with male mice of proven fertility. Starting 1 wk after the last injection, the females were placed with fertile males and mating was determined by observing a vaginal plug. The animals were killed 7–9 days after mating and the numbers of viable-appearing implants were counted. The results were expressed as average number of viable implants obtained per mated mouse.

In vitro culture of preimplanting embryos

Anti-GA-1 monoclonal antibodies and antisera were used in two different studies: (1) treatment of sperm before artificial insemination of superovulated-non-immunized rabbits to evaluate effects on fertilization and in vitro cleavage of the fertilized ova, and (2) antibody incubation of fertilized ova from normal-mated superovulated rabbits to study direct effects on cleavage in vitro. The rabbits were killed 17 to 28 h after breeding, the oviducts excised and flushed with embryo culture medium. Embryo culture medium consisted of Hams F-10, 15% serum, 100 μg penicillin and 100 μg streptomycin. Two- and four-cell fertilized eggs retrieved from rabbits artificially inseminated with treated sperm were incubated in 3 ml of embryo culture medium containing rabbit normal serum in organ culture dishes (Falcon 3037). Naturally mated female rabbits provided zygotes and two-cell

fertilized ova for incubation in Ham's F-10 medium containing immunoglobulin fractions or whole antisera. Immunoglobulin fractions of ascites were salted out using 50% saturated ammonium sulfate, followed by dialysis against repeated changes of phosphate-buffered saline, and finally Ham's F-10 medium. The protein concentration was adjusted to 5 mg/ml. The basic embryo incubation medium consisted of Ham's F-10, antibiotics and contained either 10% rabbit normal serum and 20% immunoglobulin fraction of the monoclonal antibodies or 25% rabbit preimmune control serum or rabbit immune sera. The incubation was performed in 200-μl drops (8–12 ova) of medium covered with mineral oil in Petri dishes (Falcon 1008). All incubations were done in a humid atmosphere of 5% CO_2 in air at 37°C for 3–5 days.

Statistical analysis

Significance of differences was based on Chi-square analysis or unpaired Student's *t*-test.

Results

Immunization of female rabbits with purified GA-1 significantly reduced fertility based on the ratio of viable-appearing 9-day implants to corpora lutea (Table 1). There was reduction in fertility also, in the rabbits immunized with the detergent-solubilized rabbit testis (DOC-RT). A representative electrophoresis gel of the nonreduced GA-1 used in immunization is given in Fig. 1.

Female mice immunized with GA-1 showed an inhibition of fertility as expressed by average number of viable appearing implants at 7–9 days after mating (Table 2). In all of the four trials performed, there was a significant reduction of fertility as compared with that of controls, although the s.c./i.m. injections (Trials 3 and 4) appeared less effective. Immunization with the same amount of other sperm/testis antigens, 7C6.3 Ag or 8F/G Ag, did not reduce fertility as compared to adjuvant-only injected mice. Injection of the DOC-RT, from which GA-1 and the other antigens have been isolated, also resulted in reduced fertility.

TABLE 1

Fertility at 9 days post-insemination of does immunized against germ cell antigen (GA-1) of the rabbit

Treatment	No of rabbits	No of implants/CL [a]	Percent implants	Modal ELISA titers
Rabbit testis extract (DOC-RT)	3	13/30	43 3 [b]	100
GA-1	6	20+1 deg/55	36.3 [b]	6400
Adjuvant	3	24/28	85.7	< 25

[a] CL, corpora lutea.

[b] Means differ significantly from control mean ($P < 0.05$)

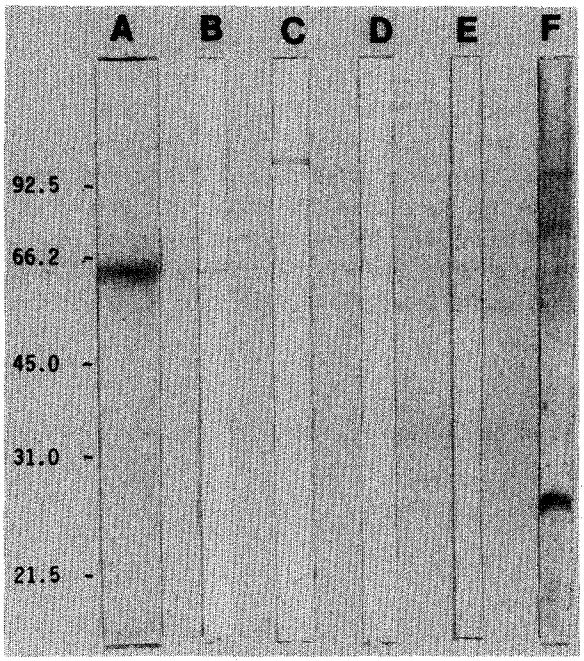

Fig. 1. Lane A, SDS-gel of GA-1 stained with Coomassie blue. 10 μg protein loaded in lane. Lanes B–F, Western blots of DOC-RT gels reacted with different antisera: B, mouse anti-GA-1; C, mouse anti-8F/G; D, mouse anti-7C6.3; E, rabbit anti-GA-1; F, rabbit anti-DOC-RT. Molecular weight standards ($\times 10^3$) are indicated to left of lane A.

TABLE 2

Fertility of female mice immunized against GA-1 and control germ cell antigens from the rabbit

Trials	Number	Control groups			Experimental groups	
		Adjuvant	7C6.3 Ag	8F/G Ag	Rabbit testis	GA-1
1&2	Mice	16	5	–	5	12
	Mean implants ± S.D.	8.0 ± 3.4	7.4 ± 2.2	–	1.0 ± 2.2 [a]	1.2 ± 1.6 [a,b]
3&4	Mice	16	14	8	–	17
	Mean implants ± S.D.	10.1 ± 3.9	8.4 ± 2.7	10.8 ± 4.6	–	3.8 ± 3.4 [a,b]
	Modal ELISA titers	< 20	< 20	< 20	60 [c]	1620

Injections via i.p. route for Trials 1 and 2 via s.c./i.m. for Trials 3 and 4.

[a] Means differ from means of control groups ($P < 0.01$).

[b] Trials 1 and 2 mean differs from Trials 3 and 4 mean ($P < 0.05$).

[c] Value for pooled serum samples from Trials 1 and 2.

Antisera and uterine fluids (UF) from rabbits and antisera from mice collected at different times after immunization with GA-1 were negative in the sperm immobilization technique (SIT) and gelatin agglutination technique (GAT). Anti DOC-RT antisera from both species exhibited against rabbit sperm GAT titers ranging from 1 : 64 to 1 : 512. None of the mouse nor rabbit antisera had detectable immobilizing activity against mouse epididymal sperm except for the serum samples from mice immunized with DOC-RT that showed low SIT titers of 1 : 4 or 1 : 8. The immune sera and UF appeared to be monospecific and bound to a single band in Western immunoblot of SDS gel (unreduced) involving deoxycholate-solubilized rabbit testis (Fig. 1). Sera from mice immunized with the control antigens 8F/G and 7C6.3 reacted with bands of approximately 100 kDa and 75 kDa, respectively, whereas rabbit antiserum against DOC-RT detected three bands at approximately 96, 82 and 28 kDa. The indirect immunofluorescence technique indicated that serum antibodies against GA-1 were bound to the acrosomal cap of methanol-fixed sperm and the plasma membrane of viable sperm. The immune sera anti-GA-1 antibody activity was not removed by absorption with somatic tissues.

Anti-GA-1 antibody activity was detected utilizing goat antibodies specific for α and γ chains of rabbit Ig in both the IgA and IgG components of UF by immunoblotting, indirect immunofluorescence and the ELISA. At 1 : 10 dilution of UF the IgA antibodies gave a strong reaction in immunofluorescence with acrosomes of fixed sperm and plasma membrane of viable sperm, whereas the IgG antibodies gave similar but weaker reactions. Reacting with anti DOC-RT UF at a 1 : 10 dilution resulted in both Ig classes of antibodies staining strongly the acrosome, postacrosomal area and main tail piece of fixed sperm and the plasma membrane of viable sperm. Titers of anti-GA-1 antibodies as measured by the ELISA were low ($\sim$ 1 : 100) for sera from rabbits immunized with DOC-RT in comparison to modal titers (1 : 6400) of the GA-1-immunized rabbits (Table 1). There was lack of a significant correlation (0.17) between antibody titer and measure of fertility in these rabbits. In mice, however, results from Trials 3 and 4 (the only trials in which individual serum titers were determined) revealed that antibody titer of the GA-1-immunized group was negatively associated ($r = -0.50$, $P < 0.05$) with fertility (Table 2). The ELISA data also indicated the specificity of GA-1 in comparison to the other two antigens used as controls in mice.

The ELISA with the UF of the two GA-1-immunized rabbits gave titers of 1 : 60 and 1 : 180 for antibodies of the IgA class and 1 : 20 for IgG antibodies. No antibodies of the IgA class were detected in the sera of these two rabbits, whereas the IgG titers were 1 : 540 and 1 : 1620. Neither serum nor UF reacted with oocytes, two-cell embryos or with 4-day blastocysts in the indirect immunofluorescence technique.

The reduction in fertility was not due to inhibition of fertilization rates. The percentage of fertilized eggs obtained from female rabbits inseminated with sperm treated with anti-GA-1 antibodies (monoclonal or polyclonal) was not significantly different from that of controls (Table 3). The percentage of two- and four-cell embryos obtained from these rabbits that developed into blastocysts, however, was significantly less than that of embryos obtained from rabbits inseminated with

TABLE 3

In vitro development after 5 days of fertilized eggs retrieved from rabbits inseminated with sperm treated with anti-GA-1 antibodies

Antibody/sperm treatment	Number		Percent fertilized eggs attaining		
	Rabbits	Fertilized eggs/ total No. eggs (%)	2–4 cell	8–32 cell	Blastocyst
Monoclonals					
Control (7C6-IgG)	1	19/22 (86.4)	21	26	53 [b]
Anti-GA-1 IgG	2	41/50 (82.0)	54	36	10 [c]
Rabbit serum					
Preimmune	3	40/43 (93.0)	2	10	88 [d]
Immune No. 4	3	48/67 (71.6) [a]	29	44	27 [e]
Immune No. 9	3	89/107 (83.2)	17	27	56 [f]

[a] One rabbit had an immature reproductive tract and only 1 of 15 eggs retrieved was fertilized.
b vs. c, $P < 0.01$; d vs. e, $P < 0.05$; d vs. f, $P < 0.05$.

TABLE 4

Effects after 4 days of in vitro incubation of normally fertilized rabbit zygotes in the presence of antibodies against GA-1

Treatment group	Number zygotes incubated	Percent zygotes attaining [b]		
		2–4 cell	8–16 cell	≥ 32 cell
Monoclonal antibodies				
Control (7C6.3) IgG	78	17	44	20
Anti-GA-1 IgG	54	20	33	28 [a]
Rabbit serum				
Preimmune serum (10%)	25	16	36	24
Preimmune serum control (25%)	19	16	53	26
Rabbit 4 immune serum [c]	17	12	36	19 [a]
Rabbit 9 immune serum [c]	17	12	30	35 [a]

[a] Nonsignificant vs. respective controls ($P > 0.05$).
[b] Some zygotes failed to cleave, resulting in less than 100% totals for the three columns of cleaved embryos.
[c] ELISA titers: No. 4, 6400; No. 9, 1600.

sperm treated with the respective control monoclonal antibody (7C6.3) or preimmune rabbit serum. There was no direct significant effect of anti-GA-1 antibodies on normal fertilized eggs incubated in vitro with the different antibodies (Table 4).

Discussion

Our results indicate that active immunization of female rabbits and mice with the 63-kDa germ cell antigen (GA-1) of the rabbit results in significant inhibition of fertility. In contrast, immunization with the same amounts of two other sperm-specific antigens, 7C6.3 Ag and 8F/G Ag, did not inhibit fertility. Although we

cannot completely exclude the possibility that a contaminating antigen(s) is inducing the antifertility effects observed after immunization with GA-1, it appears unlikely based on the following observations made with polyclonal antisera from mice and rabbits: a single band of approximately 63 kDa was detected on Western blots, plasma membranes of viable sperm and acrosomes of fixed sperm reacted in indirect immunofluorescence, and sperm treatment induced post-fertilization effects without interfering with fertilization. These are effects identical to those observed with the monoclonal antibodies against GA-1. Though we have not utilized different antigen levels nor looked extensively at the kinetics of the immune response, our objective was to establish that active immunization of females with GA-1 would induce infertility. In addition, a significant negative, albeit moderate, correlation was observed of antibody titer with fertility in mice. We also observed that rabbits responded against GA-1 with secretion of local antibody of the IgA class as well as IgG antibodies, suggesting that sperm entering the reproductive tract of the immunized female would be exposed to antibodies of both classes. The IgA antibodies of uterine origin reacted comparably to serum antibody of the IgG class in the different assay systems. The ip route of immunization with DOC-RT and GA-1 resulted in a greater reduction of fertility in mice than the sc/im route. This may have been due to a greater local immunity in the reproductive tract as serum GA-1 antibody titers did not appear to differ by route of injection or the ip injections caused greater peritoneal adhesions and inflammatory reactions that interfered with fertility. However, gross observation at necropsy did not suggest differences between the DOC-RT, GA-1 and control 7C6.3 groups in abdominal reactions. These reductions of fertility in the mice injected ip with DOC-RT and GA-1 also appeared to be greater than that seen in the homologous rabbit system. However, examining fertility rates of mice and rabbits that were immunized against GA-1 by s.c. and i.m. injections revealed comparable reductions of approximately 60% relative to respective control groups. Induction of infertility after immunization with DOC-RT was probably a result of other antigens as we estimate from immunoaffinity column data that GA-1 constitutes less than 1% of the DOC-RT protein and, in addition, anti GA-1 antibody titers were low in these animals and no reaction was observed at the 63 kDa range in Western blotting. The observation that anti-GA-1 antibodies do not block fertilization is comparable to those obtained in earlier studies with rabbits inseminated with sperm treated with monoclonal or polyclonal antibodies against GA-1 (Naz et al., 1983, 1984b). These results were confirmed further by in vitro experiments in mice in which the fertilization rates were: monoclonal antibody 8C10.5, 64% (16/25); polyclonal antiserum, 44% (14/32); both of which were not significantly different from that of controls, 79% (40/50). The fertilization rates observed in the presence of MA-24, a monoclonal antibody which has been reported to inhibit fertilization (Naz et al., 1984a), were 0% (0/30) in these experiments (M. Hamilton, R.K. Naz and A.C. Menge, unpubl. data). Likewise, two anti GA-1 antisera and the monoclonal antibody (8C10.5) were without significant effects on penetration by capacitated human sperm of zona-free hamster eggs in comparison to control samples. These antibodies show binding to the surface of capacitated human sperm by indirect immunofluorescence (unpubl. data).

That anti-GA-1 antibodies are not directly cytotoxic or inhibitory to preimplantation embryos was demonstrated by a lack of immunofluorescent staining of blastocysts by immune serum and uterine fluid samples and also by a lack of a significant effect on cleavage rates of fertilized eggs incubated in the different antibody samples. Fertilized eggs obtained from does inseminated with sperm incubated with anti-GA-1 antibodies, however, exhibited a significant inhibition of normal cleavage and development either in vivo or upon in vitro incubation. These data suggest that sperm have to carry these antibodies into ova during fertilization to exert the antifertility effect. The post-fertilization pre-implantation antifertility effect was apparently not due to parthenogenic activation or to polyspermy (Naz et al., 1984b). These antibodies may be affecting some extranuclear factor(s) on the sperm plasma membrane which is necessary for viable embryo development or cleavage. The monoclonal or polyclonal antibodies to GA-1 showed a reaction with human sperm (Naz et al., 1984b). Thus, GA-1, along with other sperm-specific antigens, namely LDH-C_4 (Goldberg et al., 1981), RSA-I (O'Rand, 1981) and FA-I (Naz et al., 1984a; Naz, 1986; Naz et al., 1986), are potential candidates for the development of an antisperm contraceptive vaccine and should help to define the mechanisms underlying fertilization and successful conception.

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