Antiacrosin antibodies and infertility. II. Gene immunization with human proacrosin to assess the effect of immunity toward proacrosin/acrosin upon protein activities and animal fertility

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Objective: To assess the effect of antiacrosin antibodies upon proacrosin/acrosin activities and animal fertility.
Design: Prospective study.
Setting: Basic research laboratory.
Patient(s): A gene immunization (GI) model was developed; mice were injected with the sequence encoding human proacrosin (h-proacrosin), cloned in an expression vector.
Intervention(s): Subcloning of h-proacrosin in a eukaryotic expression vector (promoter, CMV; leader sequence, α-1 antitrypsin; pSF2-Acro); GI of female mice with this plasmid.
Main Outcome Measure(s): The following parameters were evaluated: [1] adequate conditions for GI protocols, [2] humoral response to GI with pSF2-Acro, [3] protein regions recognized by the antibodies, and [4] effect of antibodies upon proacrosin/acrosin–ZPA binding and amidase activity, and animal fertility.
Result(s): Conditions of female mice GI with the proacrosin sequence were established (plasmid purification with anion exchange chromatography and 40 μg of pSF2-Acro per dose) to trigger an immune response, reaching maximum levels at week 9 after the first injection. Antibodies produced by GI recognized human and mouse sperm acrosin systems, inhibited human proacrosin/acrosin interaction with recombinant human ZPA and protease activity, and negatively affected mouse IVF and early embryonic development. In addition, mice immunized with SF2-Acro exhibited a significantly lower size of fetuses.
Conclusion(s): Antiacrosin antibodies developed by using GI inhibit human proacrosin/acrosin activities and impair mouse fertility. (Fertil Steril® 2009;91:1256–68. ©2009 by American Society for Reproductive Medicine.)

Key Words: Human, gene immunization, spermatozoa, proacrosin/acrosin system, antisperm antibodies, ZP binding and amidase activity, IVF and early embryonic development, fertility

Mammalian fertilization comprises a set of molecular interactions between the spermatozoon and the unfertilized oocyte, giving rise to a new zygote. Initially, spermatozoa interact with the extracellular matrix that surrounds the oocyte, the zona pellucida (ZP); this interaction triggers acrosomal exocytosis, a process by which fusion of the plasma and outer acrosomal membranes occurs, with a concomitant release of the acrosomal contents. Acrosome-reacted spermatozoa penetrate the ZP, reach the perivitelline space, and fuse to the oocyte plasma membrane (1, 2). During fertilization, spermatozoa expose protein components to the female immune system, some of which are specific to the male gamete and may be capable of triggering an immune response. This response of the immune system may naturally occur and, in human beings, may compromise fertility (3–5).

The development of antibodies toward specific proteins has been used extensively in animal models and human beings as a tool to assess the role of sperm proteins as putative receptors during fertilization. By using this approach, antibodies have been used to characterize several sperm proteins at biochemical and molecular levels, as well as to test their ability to block sperm–oocyte interaction (i.e., SP17 [6]; P34H [7]; β1,4-galactosyltransferase [8]; PH20 [9]; FA-1 [10]; sp56 [11]; SP-10 [12]; SLIP-1 [13]; YLP[12] [14]; LDH-C4 [15]; SED1 [16]; p66 [17]; SPAG9 [18]; IAM38 [19]; and NAG [20]).

In addition to these findings, reports have shown reduced in vivo fertility in female animals that were immunized with

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sperm antigens, suggesting the negative impact of the antibodies toward animal fertility (e.g., PH20 [21], FA-1 [22], SP17 [23], sp56 [24], and YLP(12) [25]). Moreover, reactive immunoglobulins toward some of these proteins have been identified in sera from subfertile women (26), supporting their immunogenicity and increasing concerns about their potential deleterious effect upon fertilization.

The protein system proacrosin/acrosin has been studied extensively in spermatozoa. Acrosin (EC 3.4.21.10) is a serine protease that is found in all species studied (27–29); it is stored in the acrosome as its zymogen, proacrosin, and it is activated to the mature enzyme and released during sperm acrosomal exocytosis (30, 31). During proenzyme activation, processing of both carboxy- and amino-terminal protein ends appears to take place (32–34). Evidence supports the participation of the proacrosin/acrosin system in fertilization, by assisting in the release of other acrosomal components, as well as by recognizing and binding to ZP glycoproteins (35–37).

Reports elsewhere have suggested the negative effect of circulating antiacrosin antibodies on proacrosin/acrosin activities (38, 39) and upon animal fertility (40–42). In agreement with this evidence, our group has described the presence of significant levels of antiacrosin antibodies in 19% of sera from women consulting for infertility. Immunoglobulins from reactive sera toward proacrosin/acrosin inhibited binding to recombinant human ZP glycoprotein ZPA (hZPA)[1] as well as proenzyme activation and amidase enzyme activities (43). On the basis of these observations, the identification of circulating antiacrosin antibodies in women's sera may be relevant regarding their potential effect upon those women's fertility. Because patients' sera that is immunoreactive to proacrosin/acrosin most likely contain immunoglobulins to other sperm antigens involved in fertilization, they cannot be used to determine their effect toward acrosin functions and unequivocally associate these results with their impact upon sperm–egg interaction. To overcome these limitations, an animal experimental model was designed to generate antiacrosin antibodies; this model was used to assess the effect of the antibodies on proacrosin/acrosin activities and upon sperm performance in IVF and early embryonic development (EED), as well as on in vivo animal fertility.

Up until recently, protocols for active immunization have involved the identification, purification, and inoculation of the native protein and, more recently, production of recombinant proteins, mainly in bacteria, followed by their purification and injection using standard protocols. In the early 1990s, a new method was introduced (44–46) that allows the induction of immunity toward a protein after injection of the DNA encoding the polypeptide sequence; this procedure was called *gene immunization* (GI). Compared with traditional methods of protein injection, GI has proved to be advantageous, because it is applicable to any protein whose sequence is known; because plasmid DNA purification involves the use of convenient standardized procedures to prepare the immunogen, and because it avoids isolation and purification of the protein antigen, as well as protein denaturation during purification (47–49). To the present time, this procedure has been used extensively for the development of immunity toward many proteins from bacteria, parasites, and viruses as well as toward tumor antigens and modulators of the immune system (50–52). More recently, some reports have been published describing its effectiveness in the development of antibodies toward oocyte and sperm antigens (ZPB [53], ZPC [54], PH20 [55], LDH-C4 [56], YLP12 [57], SPAG9 [58], and FA-1 [59]).

On the basis of these previous findings, the aim of this study was to test the efficacy of the GI system for the development of immunity toward the proacrosin/acrosin system in a murine model; parallel experiments were performed, injecting recombinant human acrosin Rec-30 (36) and a plasmid with the sequence encoding the human proenzyme. In addition, the GI model was partially characterized (protocol optimization and assessment of humoral response along time). Finally, evaluations were performed to determine the effect of the antibodies generated by GI upon protein activities and to evaluate their impact upon in vivo fertility, as well as IVF and EED.

MATERIALS AND METHODS

Unless specified, chemicals were of analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO). Electrophoretic reagents were from BioRad (Hercules, CA), and molecular biology reagents were from Qiagen (Hilden, Germany), Invitrogen (Carlsbad, CA), and New England Biolabs (Beverly, MA). The monoclonal antiacrosin antibody AcrC5F10 was purchased from Biosonda (Santiago, Chile).

The pSF2 eukaryotic expression vector used for genetic immunization protocols (60) contains the cytomegalovirus (CMV) promoter and a sequence for the α-1 antitrypsin secretory leader. A human proacrosin (h-proacrosin) cDNA clone was carried in the plasmid human acrosin (PHA) plasmid (61). For all procedures, standard molecular biology protocols were used (62).

Gametes and Animals

Protocols performed throughout the study using human samples were approved by the Ethical Committee of the Instituto de Biología y Medicina Experimental (Buenos Aires, Argentina). Semen samples were obtained from normozoospermic donors, according to World Health Organization standards (63). Only samples with >90% live spermatozoa, 75% progressive motile cells, and >14% percentage of normal sperm forms (Kruger strict criteria) were included in the study.

[1] Also named as ZP2 (Symbol report: ZP2. Human Genome Organization (HUGO). Gene Nomenclature Committee. April 25, 2005. World Wide Web. URL http://www.genenames.org/data/hgnc_data.php?hgnc_id=13188

Animals were treated in compliance with regulations for the protection of animal rights; animal handling was performed in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals (64). We selected BALB/c female mice for GI and for in vivo fertility studies. We used CF1 animals for epididymal sperm recovery (immunocytochemistry and preparation of protein sperm extracts), for IVF, and for EED protocols.

Production and Purification of Recombinant Human Acrosin Proteins and Recombinant hZPA

Recombinant h-proacrosin (Rec-40) and *N*-terminal fragments (Rec-30, Rec-20, and Rec-10) were obtained by using the pET-22b prokaryotic expression vector, as described elsewhere (36). Recombinant hZPA was produced and purified as described elsewhere (65).

Assessment of Proacrosin–ZPA Binding and Acrosin Enzymatic Activity

Binding of proacrosin to recombinant hZPA was evaluated as recently described (37). Assessment of proacrosin activation and acrosin amidase activity was performed essentially as described in the accompanying article to this one (43). The rate of inhibition was determined by calculating $[1-(OD_{Ig}/OD_n)] \times 100$, where OD_{Ig} are the optical density (OD) units obtained in presence of immunoglobulin, and OD_n are the OD units obtained in the absence of immunoglobulin.

Subcloning of Human Proacrosin in a Eukaryotic Expression Vector Used for GI

The selected strategy involved cloning of the sequence encoding h-proacrosin in the *Bam*HI–*Eco*RI sites of the pBluescriptII KS phagemid (Stratagene, La Jolla, CA), which was excised with *Bam*HI and *Hin*dIII and ligated into the pSF2 eukaryotic expression vector. The proacrosin coding sequence was cloned in the first vector in two steps, the first one involving a PCR procedure using PHA as template to generate a *Bam*HI–*Eco*RI fragment encoding amino acids 1–60 of the proenzyme (5′COMBO primer: TTGGGATCC ATATGAAAGATAACGCCACGTGTGAT; 3′ECO primer: TCGTGAATTCAGCAAGCTGCCT). In the second step, a 1,150-bp *Eco*RI–*Eco*RI fragment of PHA (spanning nucleotides 234 to 1266 of the complementary DNA (cDNA) and 118 additional nucleotides 3′ from the proacrosin stop codon [36]) was subcloned in the *Eco*RI site of pBluescriptII carrying the 5′ coding region of proacrosin from the PCR fragment. The h-proacrosin sequence was excised on the *Bam*HI and *Hin*dIII restriction sites and ligated in the pSF2 vector; the expression vector containing the h-proacrosin cDNA was named pSF2-Acro. The clone selected for GI purposes was subjected to nucleotide sequence analysis to verify the sequence of the PCR fragment, as well as the insert reading frame. Sequence comparisons were performed by using the Basic Local Alignment Search Tool (66) and Entrez software (67). Plasmid DNA purification was performed by using a standard phenol extraction protocol or by ionic exchange chromatography, using a commercial kit (Plasmid DNA purification kit, Qiagen).

Immunization Protocols

The BALB/c (5–7 wk of age) female mice were inoculated IM in the quadriceps muscle with 10, 20, or 40 μg of the plasmids pSF2-Acro and pSF2 (control) in a final volume of 50 μL, with a 27-G needle. In parallel, a group of animals were injected with the recombinant fragment of h-acrosin produced in bacteria, Rec-30 (36) (control: same volume of vehicle); protein injections were intraperitoneal, with Freund's complete adjuvant used for the first dose (10 μg of Rec-30) and incomplete adjuvant, for the following boosts (5 μg of Rec-30). Details on the number of animals included in each experiment are provided in Results. In all cases, injections were repeated four times, every 3 weeks. Before each injection, animals were treated with 10% tribromoethanol (120 μg per g of body weight) (68). To test for the immune response to protein and DNA injections, blood samples were drawn by retrobital puncture into capillary tubes before each inoculation. Sera were recovered after blood centrifugation and stored at −20°C until used. Long-term response was assessed until 6 months after the beginning of the immunization protocol with plasmid DNA. In all cases, mice were bled by cardiac puncture before being killed, and sera were stored at −20°C until further analysis.

Evaluation of the Immune Response

Enzyme-linked immunosorbent assay Serum IgG antibody levels against h-proacrosin were assessed by the ELISA-Acro, as described in the accompanying article to this one (43), using 0.7 μg of purified recombinant proacrosin Rec-40 as antigen and 1:100 dilutions of mouse immune sera. At the time of highest response, 1:100 sera dilutions were tested by using *N*-terminal truncated acrosin proteins Rec-30, Rec-20, and Rec-10 (36). Antiacrosin antibody titers were assessed by serial dilutions between 1:100 and 1:102,400. The presence of antibodies was detected by using a system containing biotinylated goat antimouse IgG (B6649, Sigma), diluted 1:2,000 in blocking buffer, and a developing system with extravidin-alkaline phosphatase conjugate and disodium *p*-nitrophenyl phosphate added as substrate. Readings were performed at 405 nm in a microplate reader (Emax Precision Microplate Reader; Beckman Coulter, Fullerton, CA). A positive control with monoclonal antibody AcrC5F10 (1:1,000 dilution) and a negative control for mouse preimmune serum were included in all runs. Results were expressed as the ratio between readings of optical-density (OD) units in the sample and in the preimmune sera (OD_0):(OD/OD_0).

Western immunoblotting and immunocytochemistry Immune sera from mice inoculated with pSF2-Acro were tested for the presence of reactive antibodies toward human and mouse proacrosin/acrosin proteins in Western immunoblotting and immunocytochemistry.

Acid sperm extracts of human semen samples from normozoospermic donors were prepared essentially as described elsewhere (34). Spermatozoa from cauda epididymis of adult CF1 mice were recovered from tissue sections and placed in 200-μL drops of IVF culture medium (see Mouse IVF, EED, and Mating Protocols) supplemented with a cocktail of proteinase inhibitors under oil at 37°C for 10 minutes. After tissue removal from the drop, cell suspensions were concentrated by centrifugation (300 $\times$ g for 10 min) and treated with low pH buffer, as performed with human spermatozoa. Samples for analysis were prepared in Laemmli sample buffer, boiled for 10 minutes, and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis in 10%–12% polyacrylamide gels with 0.1% sodium dodecyl sulfate under reducing conditions (69). Protein molecular weight was estimated by comparison with protein standards run in parallel (broad-range molecular weight markers, Bio-Rad: myosin, 200 kDa; β-galactosidase, 116 kDa; phosphorylase b, 97 kDa; bovine serum albumin (BSA), 66 kDa; ovoalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa; and aprotinin, 6.5 kDa). For Western immunoblot analysis, proteins were electroblotted onto nitrocellulose membranes, as described elsewhere (70). Membranes containing immobilized proteins were placed in blocking solution (5% skim milk in phosphate-buffered saline [PBS] containing 0.02% Tween 20), followed by incubation with sera from immunized mice in blocking solution; the monoclonal antiserum against purified human acrosin AcrC5F10 was used as positive control. Peroxidase-conjugated sheep anti-mouse IgG (Amersham/GE, Uppsala, Sweden) was used as second antibody and developed by using enhanced chemiluminescence (ECL kit, Amersham/GE). Blot immunostaining was performed at room temperature, with constant shaking.

For immunocytochemical studies, spermatozoa from both species recovered as described above were fixed in 2% formaldehyde in PBS for 10 minutes at room temperature (human) or for 4 minutes at 4°C (mouse); at the end of the incubation, cells were washed, and 50,000 spermatozoa were loaded onto eight-spot microscope glass slides pretreated with polylysine and were allowed to dry at 37°C. Sperm cells were washed in PBS for 5 minutes, permeabilized in methanol for 10 minutes at 4°C, incubated for 30 minutes in PBS supplemented with 4% BSA to block nonspecific binding, and placed with the mouse immune sera from animals injected with plasmids pSF2-Acro and pSF2 (control) for 1 hour at room temperature for human sperm and overnight at 4°C in a humid chamber for mouse cells. After three 5-minute washes in PBS, cells were incubated with anti-mouse IgG labeled with CY3 (Sigma; mouse spermatozoa) and fluorescein isothiocyanate conjugate (Sigma; human spermatozoa) for 1 hour at room temperature in darkness. Slides were extensively washed, mounted with Vectashield (Vector Laboratories Inc., Burlingame, CA), and observed with a Nikon fluorescence microscope coupled to an image analyzer (IPLab Scientific Imaging Software for Windows; Scanalytics Inc., Fairfax, VA).

Immunoglobulin G purification The IgG fraction from preimmune and immune mouse sera was purified by affinity chromatography with Protein A agarose CL-4B; the saline concentration was adjusted to 3.3 M with NaCl and a 1/10 volume of 1 M sodium borate (pH 8.9). Immunoglobulin elution was achieved by using 100 mM glycine (pH 3.0), and column fractions were recovered in 1 M Tris (pH 8.0) (71). The eluate was concentrated by centrifugation using a Centricon YM30 (Amicon; Millipore, Billerica, MA) up to a 10 mg/mL final protein concentration, which was assessed by using the Bradford method (72).

Mouse IVF, EED, and Mating Protocols

In vitro fertilization The CF1 (45–60 d of age) female mice were superovulated by using SC injections of 7.5 IU of pregnant mare serum gonadotropin (Sigma); 54–56 hours later, animals were injected with 7.5 IU of hCG (Sigma). Animals were killed 12–14 hours after hCG injection, and cumulus masses containing the unfertilized oocytes were harvested, freed of cumulus cells after treatment with 0.3 mg/mL of hyaluronidase for 5 minutes at 37°C, and washed in IVF culture medium to remove the hyaluronidase excess (73). Spermatozoa were recovered as indicated for the preparation of mouse cell protein extracts; to promote capacitation, sperm cells (10 million/mL) were placed in IVF medium supplemented with 0.3% BSA for 2 hours at 37°C in an atmosphere of 5% CO_2 in air. Oocytes (randomly distributed in groups of 8–15 in 50-μL drops of IVF medium) were incubated with 100,000 spermatozoa per milliliter for 6–7 hours at 37°C in an atmosphere of 5% CO_2 in air. In assays performed in the presence of antiacrosin antibodies, purified mouse IgGs were added during gamete incubation at a concentration of 8 and 80 μg/mL. Fertilization was confirmed by identification of the second polar body, as well as by presence of female and male pronucleae and the sperm tail in the oocyte cytoplasm.

Early embryonic development The CF1 (60 d of age) female mice were injected with the same dose of pregnant mare serum gonadotropin and hCG, with injections separated by 46–48 hours; female animals were then placed with adult males of proven fertility in a ratio of 1:1. Two-cell stage embryos were collected from oviducts, washed three times in prewarmed PBS, and pooled in pre-equilibrated KSOM medium (74) that was supplemented with 4 mg/mL of BSA. The embryos were randomly distributed in groups of 10 in 20-μL drops and were cultured in vitro at 37°C in 5% CO_2 in air for 72 hours (day 3). At this time, the number of blastocysts in all experimental conditions was recorded. Blastocysts were cultured for additional 24 hours to allow ZP hatching. To assess embryonic viability, the percentage of dead cells in each blastocyst was determined after embryo staining with 20 μg/mL propidium iodide, according to a procedure described elsewhere (75); 40 total cells per blastocyst was used as control reference (76). To evaluate the effect of antiacrosin antibodies upon EED and embryo hatching, two-cell embryos were incubated in the presence of 2 mg/mL of purified

immunoglobulins from mice immunized with pSF2-Acro; control values were established with embryo cultures from female animals injected with pSF2 plasmid, as well as with embryos from untreated animals placed in medium with purified mouse IgG from preimmune serum. In some cases, tests were performed in the presence or absence of complement (2% guinea pig serum). Embryonic development was expressed as the percentage of blastocysts obtained in each condition. Each assay was performed in duplicate, with 10 embryos per assay.

Mating Immunized female mice were paired with adult male mice, starting at week 9 of the immunization protocol (time of maximum immune response). Mice were monitored for detection of plugs, after which they were separated from the males; females were killed 14 days later to allow scoring of total number and weight of the pups as well as of number of resorption points in each animal.

Statistical Analysis

All experiments were repeated at least three times, and duplicate evaluations or measurements were performed in each case. Data were expressed as mean ± SEM. Statistical data analysis was performed by using Statistica for Windows, version 5.0 (Statsoft, Inc., Tulsa, OK). Data were analyzed, when appropriate, by using Fisher's exact test, Student's *t*-test, and analysis of variance, followed by multiple comparison when significant differences between mean values were found; alternatively, nonparametric tests (Mann-Whitney and Wilcoxon tests) were applied. Differences were considered significant when P was $< .05$.

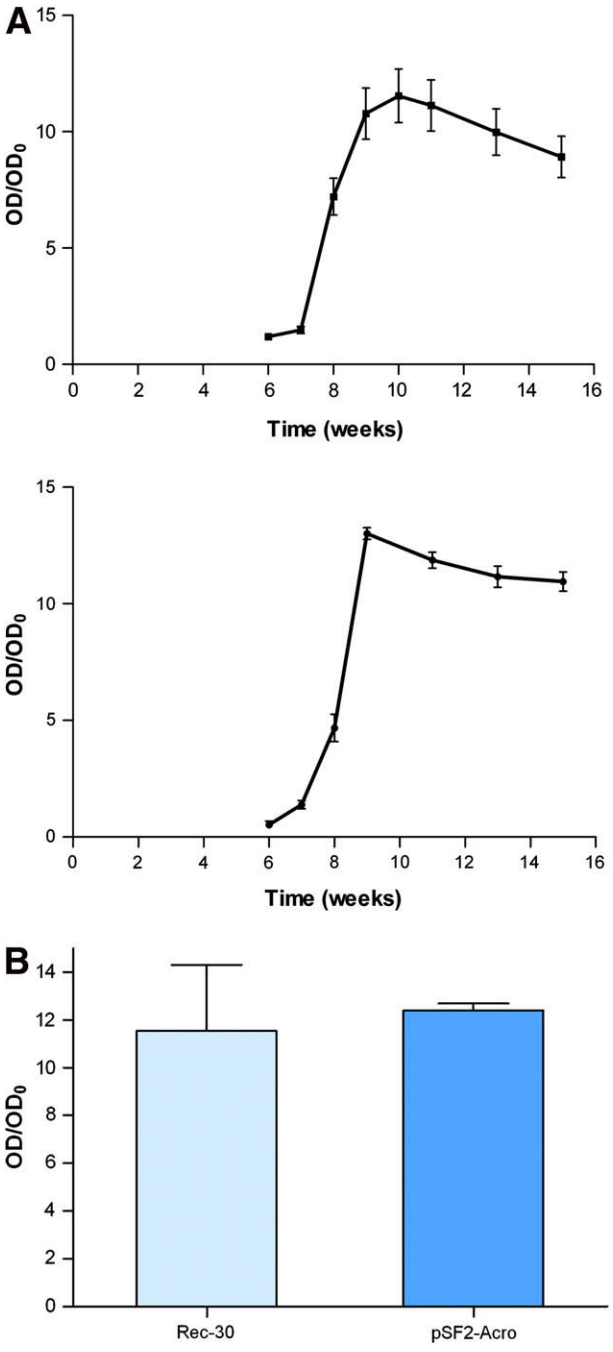
RESULTS

Development of a GI Model for h-Proacrosin to Assess the Effect of Antiacrosin Antibodies Upon Protein Activities and Participation of Proacrosin–Acrosin in Fertilization

The efficacy of using a GI model to trigger an immune response toward proacrosin/acrosin in mice was tested initially. The sequence of h-proacrosin was cloned in the eukaryotic expression vector pSF2 by using the strategy detailed in Materials and Methods. Parallel immunization protocols using injections of 40 μg of pSF2-Acro per dose or of 5 μg per dose of the Rec-30 acrosin protein gave similar humoral responses across the monitored time (Fig. 1A). A maximum OD reading for the immunoglobulins was observed at week 9 after the injection of DNA and at week 10 after protein immunization (Fig. 1B). Both methods induced high antibody titers, ranging from 1:1,600 to 1:512,000; similar humoral responses were obtained in protocols using 5 μg per dose of Rec-30 protein and 40 μg per dose of pSF2-Acro plasmid (range, 1:6,400–1:512,000). Because GI involves a fast and reasonably simple method for the production of high amounts of the plasmid immunogen encoding the proenzyme without the trouble associated with protein degradation during purification procedures, renders a sustained immune response, and allows the exposure of the

FIGURE 1

(**A**) The humoral response toward proacrosin/acrosin as a function of time. Antiacrosin antibodies in mouse injected with Rec-30 (n = 5; *upper*) or pSF2-Acro (n = 5; *lower*). (**B**) Levels of antiacrosin antibodies assessed on week 9 of the protocol. Differences were not significant within procedures ($P > .5$; Mann-Whitney test).



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entire native protein to the immune system, this procedure was selected for further studies.

A group of evaluations was first performed to select the adequate experimental conditions to develop antibodies toward the proacrosin/acrosin system. Taking into consideration that presence of endotoxins in plasmid DNA preparations has been reported elsewhere to cause a dose-dependent cytotoxic effect (77), DNA purification protocols using phenol–chloroform precipitation and ionic exchange chromatography were compared with regard to their respective immune response levels. The results revealed a significant higher immune response in animals inoculated with plasmid pSF2-Acro purified by anionic exchange chromatography than that in mice injected with plasmid purified with classical phenol/chloroform protocols (at week 9, with 40 μg per dose of pSF2-Acro, $OD/OD_0 = 13.04 \pm 1.00$ vs. $OD/OD_0 = 2.52 \pm 0.83$, respectively; $P<.05$; Mann-Whitney test). Thus, the first procedure was selected for plasmid purification in all experiments presented in this article.

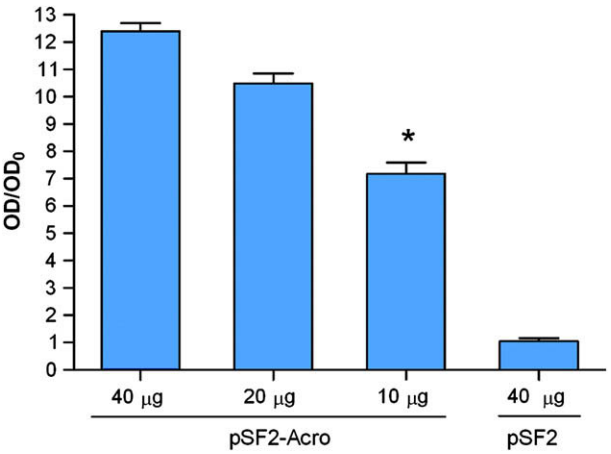
A dose of plasmid pSF2-Acro that gave a high humoral response was determined by inoculating animals with different amounts of pSF2-Acro plasmid per dose. A similar time course was observed with all doses tested (data not shown), but a significantly higher response was obtained in animals injected with 20 and 40 μg per dose of pSF2-Acro ($P<.05$; analysis of variance) than in those immunized with 10 μg per dose of the plasmid (evaluations performed at wk 9 of the immunization protocol; Fig. 2). Parallel experiments performed in mice injected with 40 μg of pSF2 showed OD values similar to those obtained with preimmune sera, with an $OD\text{-}OD_0$ ratio range between 0.98 and 1.3 at week 9 from the first injection.

The immune response to plasmid DNA was monitored between weeks 6 and 18; a high response was observed in all animals injected with pSF2-Acro, with a maximum response in week 9 from the first injection and sustained during at least the following 6 weeks (mean OD/OD_0 values of group immunized with 40 μg of pSF2-Acro, week 9: 13 ± 1.0; week 16: 10.9 ± 0.9). The response to injection of pSF2 remained low throughout the immunization protocol. On the basis of these findings, injections with 40 μg of plasmid DNA (pSF2-Acro and control pSF2) were selected for the standard immunization protocols. Considering that the protocol for injection involved IM inoculation of the plasmid DNA, immunohistochemical analysis of the muscle in which the injection is performed was performed with the aim of localizing the protein; however, no signal for proacrosin/acrosin could be detected (data not shown).

The long-term humoral response was monitored for ≤ 6 months after the initiation of the immunization protocol. Evaluations performed in serum samples from five animals injected with 40 μg and from two animals injected with 20 μg of pSF2-Acro plasmid showed a trend toward a decrease in the titer in all cases, although a signal still remained in week 24 in all sera tested, ranging from 35% to 65% of the maximum value scored on week 9 (data not shown).

FIGURE 2

Antiacrosin antibodies in female mice injected with 10, 20, and 40 μg of pSF2-Acro plasmid DNA; response at week 9 of the immunization protocol. *Significant differences were observed between results for protocols with 10 μg of plasmid compared with those using 40 or 20 μg ($P<.05$, analysis of variance; n = 6 in each group).



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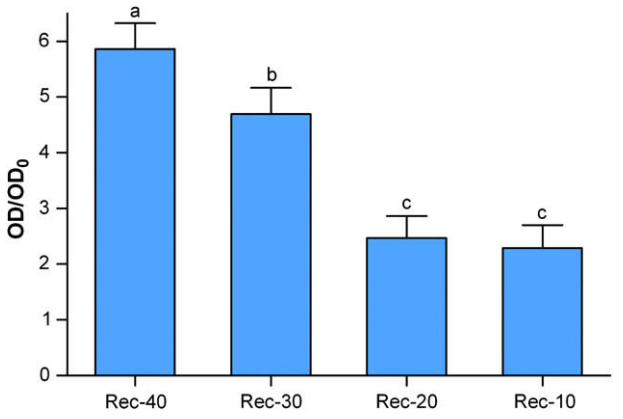
Reactivity of antiacrosin antibodies produced by GI toward different protein regions was assessed in the ELISA-Acro test using Rec-40 (recombinant human proenzyme) and truncated recombinant proteins Rec-30, Rec-20, and Rec-10 as antigens. In all sera tested, the highest response was obtained with Rec-40 as antigen; Rec-30 retained 80% of the signal observed with Rec-40 ($P<.05$), whereas ODs for Rec-20 and Rec-10 were 42% and 39% of that for Rec-40 ($P<.05$; Fig. 3).

Altogether, the results from these studies suggest the effectiveness of the GI protocol, with the plasmid carrying the sequence encoding h-proacrosin in the development of an adequate and specific immune response toward proacrosin/acrosin in mice.

Ejaculated human and epididymal mouse spermatozoa were subjected to immunocytochemical analysis with sera from animals inoculated with plasmid pSF2-Acro; control experiments were performed using sera from animals injected with pSF2. As shown in Figure 4A and B, a specific signal for proacrosin/acrosin located on the acrosomal region of sperm cells was obtained in both species. Western immunoblot analyses of sperm protein extracts from both species showed the ability of the antiacrosin antibodies to recognize protein forms of the proacrosin/acrosin system in human beings (53 kDa; proacrosin) and mice (48 kDa, proacrosin; Fig. 4C). These studies indicated that antiacrosin

FIGURE 3

Humoral response to h-proacrosin (Rec-40) and *N*-terminal truncated proteins Rec-30, Rec-20, and Rec-10 in animals receiving pSF2-Acro. Results are presented as percentages of the signal relative to that obtained with Rec-40. Different *lowercase letters* refer to significant differences ($P<.05$; analysis of variance). Evaluations were performed on sera from 14 animals.



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antibodies generated by GI recognize the native human and murine proacrosin/acrosin proteins from sperm cells.

Effect of Antiacrosin Antibodies Produced by GI Upon Proacrosin–Acrosin Activities and Sperm Fertilizing Ability

To assess the effect of the antiacrosin antibodies obtained by GI of h-proacrosin upon the interaction between the human proenzyme and ZP glycoproteins, purified IgGs from the immunized mice were added at 1 and 10 μg/mL final concentrations to the Rec-40–rechZPA binding assay; control assays were run with equal amounts of IgGs from animals injected with pSF2. As shown in Table 1, presence of antiacrosin antibodies resulted in a decrease of recombinant hZPA binding, reaching in all cases approximately 50% inhibition levels at the highest concentration tested.

Incubation of protein sperm extracts under conditions to promote proacrosin activation in the presence of IgGs from mice injected with pSF2-Acro resulted in an inhibition, although weak, of the acrosin amidase activity in two of the four sera tested (Table 1); similar results were obtained when the IgGs were added after activation of the proenzyme pool (data not shown).

On the basis of these results, experiments were performed to assess the effect of the antiacrosin antibodies upon sperm–oocyte interaction. To evaluate whether the presence of circulating antiacrosin antibodies in mice immunized with pSF2-Acro had an effect upon in vivo animal fertility, female animals were mated at week 9 from the beginning of the GI protocol with adult male mice of proven fertility and were evaluated as detailed in Materials and Methods. A significant decrease in the average fetus size ($P<.01$) and a trend toward a decrease in the number of pups were observed in animals that were injected with pSF2-Acro, compared to controls (Table 2).

To analyze the effect of the antibodies upon in vitro sperm–oocyte interaction, mouse IVF assays were performed, with some IgGs from the immune sera that were produced by GI with pSF2-Acro added to the gamete incubation drop at a final concentration of 8 and 80 μg/mL. As shown in Figure 5, presence of some of the IgGs (IgGI-1, IgGI-2, and IgGI-3) toward h-proacrosin/acrosin resulted in a significant decrease in the percentage of fertilized oocytes ($P<.05$) when antibodies were added at the highest concentration tested; presence of IgGs from pSF2 control plasmid (IgGI-11; similar results with additional 2 sera, data not shown) had no effect upon fertilization. Inhibition could not be attributed to either sperm agglutination or inhibition of sperm motility (data not shown).

In addition to the effect of antiacrosin antibodies upon IVF, studies were performed to evaluate whether these antibodies could have an effect upon in vitro EED. Two cell-mouse embryos were cultured with the antibodies in the presence or absence of complement, and the percentage of blastocysts, as well as the rate of embryo hatching, was scored. Purified antiacrosin IgGs had a slight negative effect toward both blastocyst development and hatching. Immunoglobulin G from one animal (IgG I-4) led to a complete inhibition of blastocyst development. Assessment of blastomere viability revealed a higher number of dead cells in embryos cultured with antiacrosin antibodies produced by GI (Table 3).

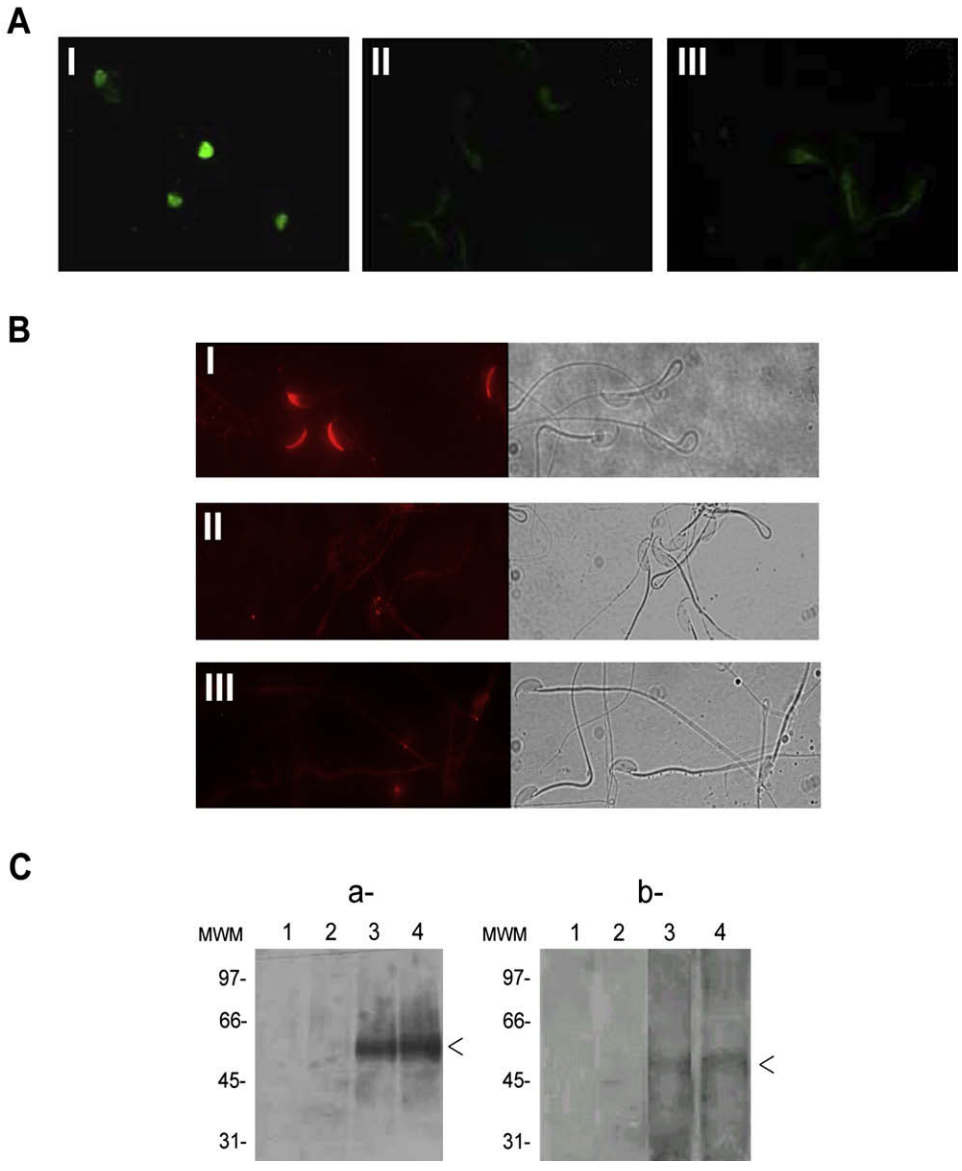
Altogether, these studies revealed the ability of antiacrosin antibodies produced by GI to inhibit h-proacrosin/acrosin ZP binding and protease activities and to interfere with mouse in vivo and in vitro fertility.

DISCUSSION

Recent studies from our group have described the identification of antibodies toward the proacrosin/acrosin system in women consulting for infertility and their effect upon the sperm proteinase activities (43). Considering the negative impact of antisperm antibodies upon gamete interaction and fertility (3–5), the present investigation was aimed at developing an animal model with circulating antiacrosin antibodies, to assess the antibodies' effect on proacrosin/acrosin activities and on in vivo and in vitro animal fertility. The studies have demonstrated for the first time the feasibility of generating an immune response toward proacrosin by DNA immunization of mice, as well as the ability of the circulating antiacrosin antibodies to recognize the human and murine native proacrosin/acrosin systems and to impair proacrosin/acrosin activities, as well as to negatively affect murine IVF and EED.

FIGURE 4

Immunodetection of proacrosin/acrosin in human and mouse spermatozoa. Immunocytochemistry of human (**A**) and mouse (**B**) sperm cells with sera from animals injected with pSF2-Acro plasmid (**AI**), with sera from controls incubated with a serum from an animal injected with pSF2 (**AII**), and with preimmune serum (**AIII**). Original magnification, ×400. (**C**) Western immunoblotting of protein extracts from human (**Ca**) and mouse (**Cb**) spermatozoa with a pool of sera from animals injected with pSF2-Acro plasmid (*lane 3*), and negative controls with sera from animals injected with pSF2 (*lane 2*), or omitting the first antibody (*lane 1*). *Lane 4,* positive control performed with monoclonal antibody AcrC5F10. MWM = molecular weight markers.



Veaute. Antiacrosin antibodies impair fertilization. Fertil Steril 2009.

Regarding the proacrosin/acrosin system, previous investigations performed in the rabbit and sheep models (40–42) characterized the response to active immunization of female animals toward the acrosomal proteinase and its impact upon fertility. Even though immunization with acrosin did not have a significant effect upon fertility in any single group, the reports revealed a positive correlation between high antibody titers toward acrosin and reduced fertility; in the study performed in rabbit, 4 of 12 animals injected did not become pregnant, 3 (75%) of which had the maximal titers of serum antiacrosin antibodies (40). Unfortunately, no further studies were published, perhaps because of limitations in obtaining

TABLE 1

Inhibition of proacrosin-ZPA binding and proacrosin activation or activity by antibodies from sera of animals injected with pSF2-Acro (IgG I-1 to IgG I-4) and pSF2 (IgG I-9 and IgGI-11).

	Inhibition of ZP-binding activity		Inhibition of acrosin amidase activity	
Serum no.	IgG concentration (μg/mL)	% (Mean ± SEM)	IgG concentration (μg/mL)	% (Mean ± SEM)
IgG I-1	10	49 ± 2	2	0
	1	39 ± 2		
IgG I-2	10	50 ± 3	2	17 ± 1
	1	48 ± 3		
IgG I-3	10	55 ± 3	2	23 ± 1
	1	50 ± 4		
IgG I-4	10	52 ± 3	2	7 ± 1
	1	53 ± 1		
IgG I-9	10	15 ± 1	**2**	0
	1	5 ± 0		
IgG I-11	10	19 ± 1	2	0
	1	12 ± 1		

Note: Data are presented as percentage of inhibition from a control without any IgG added. Binding of Rec-40 to rec-hZPA in the presence of purified IgGs and amidase activity were evaluated as indicated in Materials and Methods. IgGs were added at the beginning of the activation period.

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sufficient amounts of the native proenzyme to carry out the immunization protocols with the whole protein. Studies elsewhere have described several methods to purify the sperm zymogen of acrosin, but protein denaturation, processing by sperm proteinases, or self-activation of the proenzyme may occur during protein isolation and purification (33, 34).

The methodology of GI has been shown to be a valid alternative for the development of antibodies toward proteins of different sources, demonstrating that injection of plasmid DNA is able to induce a potent humoral immune response against the encoded genes (44–46). In the present study, this procedure was tested to assess its potential ability to induce an immune response toward the native proacrosin/acrosin system.

The h-proacrosin cDNA was subcloned in a eukaryotic expression vector with the CMV promoter, which is widely used because of its ubiquitous and high gene expression levels (78). The α-1 antitrypsin leader sequence was included to assure protein secretion; in this regard, protein exposure to the extracellular milieu by expression on the cell surface or secretion has been shown to induce more effective humoral responses than those that are obtained with intracellular antigens (79). Injection of plasmid DNA purified by anionic exchange chromatography resulted in a quite significantly higher response than that obtained with the classical plasmid phenol–chloroform purification method. These differences could be attributed to the elimination of contaminants, such as lipopolysaccharides present in Gram-negative bacteria,

TABLE 2

Inhibition of in vivo conception by circulating antiacrosin antibodies in animals injected with pSF2-Acro and pSF2 (control).

Parameter	n	Antiacrosin antibodies at time of mating (OD range)	No. of pups (average ± SEM)	Fetus average size, mm (mean ± SEM)
pSF2-Acro	4	3.5–8.5	6.5 ± 2.5	7.1 ± 2.1[a]
pSF2	4	0.9–1.5	10 ± 2.5	10.8 ± 1.6

Note: Female mice were mated with adult male mice at week 9 of the immunization protocol and killed at day 14 of pregnancy. Number and average fetus size were determined.

[a] $P<.01$, Student's *t*-test, compared with data on animals injected with pSF2.

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FIGURE 5

In vitro fertilization rates in the presence of 8 and 80 μg/mL of purified IgG from sera of animals injected with pSF2-Acro plasmid. [a]$P<.001$and [b]$P<.05$ (both, Fisher's exact test) vs. IVF rates obtained in the presence of the same amount of pSF2 IgG.

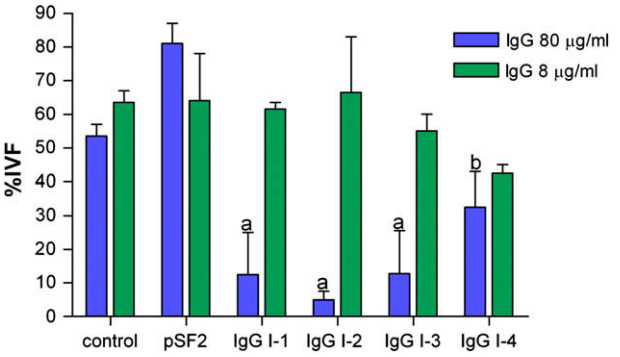

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TABLE 3

Effect of immunoglobulins from sera of animals injected with pSF2-Acro upon cell viability in mouse early embryos.

IgG added	Complement status	No. of dead cells (mean ± SEM; no. of stained blastocysts)
Group		
IgG I-1	–	7 ± 1 (7)
	+	11[a] ± 2 (6)
IgG I-6	–	29[a] ± 3 (4)
	+	43[a] ± 3 (15)
Controls		
No IgG	–	7 ± 1 (12)
Preimmune IgG	–	5 ± 2 (5)
Preimmune IgG	+	3 ± 1 (13)
pSF2 IgG	–	6 ± 1 (8)

Note: Purified IgGs (2 mg/mL) from animals injected with pSF2-Acro (IgG I-1 to IgG I-4) and pSF2 (IgG I-9 and IgGI-11) were added in the presence or absence of 2% guinea pig serum (as a source of complement) at the beginning of 2-cell embryo culture; embryos at the blastocyst stage (day 4 from beginning of culture) were recovered and stained with propidium iodide. Controls omitting IgG and incubated with preimmune IgG were included in the experimental design.

Data is presented as percentage of dead cells (value calculated by dividing the total stained [dead] cells by 40, a total number of cells estimated for embryos grown in KSOM and the ratio multiplied by 100 to obtain percentage) (76).

[a] $P<.0001$; Fisher's exact test, compared with control (preimmune IgG).

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which were reported to have a cytotoxic effect (77) and to diminish transfection efficiencies (80). The results in our study confirm these findings, emphasizing the relevance of using highly pure DNA for gene injection.

In a first set of experiments, two different antigen and delivery formulations were compared: injection of purified Rec-30 with Freund adjuvant and GI with the pSF2-Acro plasmid containing the sequence of h-proacrosin; the ability of Rec-30 (human acrosin) to raise an immune response in rabbits had been described elsewhere by our group (36). Both immunization protocols yielded similar immune responses in mice, as reflected in the comparable time evolution along the immunization protocol as well as by the presence of similar levels of antiacrosin antibodies. Additional protocols performed exclusively using GI raised an immune response toward proacrosin/acrosin in all animals injected. Long-term response studies performed with animals subjected to GI toward h-proacrosin revealed a sustained immune response 3 months after the last DNA injection dose; this time is noticeably longer than that reported for other protein antigens (81). The decrease in the signal with time suggests the reversibility of the response, although modifications in the immunization protocol would have to be tested to optimize the procedure if a complete decay in the response is expected.

Injection of DNA resulted in the expression of proacrosin in the animals, as observed with the immune response developed toward the recombinant human acrosin proteins detected in the ELISA, as well as by results in Western immunoblotting and immunocytochemical analyses of the murine and human protease systems. The lack of detection of protein expression in the animal muscle could be attributed to either limitation of the protein detection protocol used, or expression of the protein in cells that migrated to the lymph node; a weak signal observed in cells from the subcapsular sinus of the inguinal node (data not shown) would favor the latter possibility.

Antiacrosin antibodies produced by GI of the h-proacrosin cDNA recognized the human and mouse proacrosin/acrosin protein system in total sperm extracts and in the acrosome of whole cells; these results were not surprising considering the high homology between amino acidic sequence of both proteins (92%, 82%, and 51% for domains I, II, and III, respectively) (82). Antiacrosin antibodies produced by GI depicted the highest reactivity toward proacrosin residues 190–402; these observations would confirm the expression and exposure of the proenzyme to the immune system and are in agreement with the results presented in the accompanying article to this one, in which we identified circulating antiacrosin antibodies toward epitopes located along the proenzyme sequence (43), some of which may be directed to sequences of the proacrosin C-terminal region.

Antiacrosin antibodies produced by GI inhibited the interaction between recombinant h-proacrosin and hZPA. These results were similar to those obtained using sera from subfertile women immunoreactive to h-proacrosin; the purified immunoglobulins from the immunized mice impaired protein interaction at the same levels as those described with patient sera in the accompanying article to this one (43). In the present study, we were unable to identify murine immune sera that recognized only the C-terminal end of the human proenzyme; nevertheless, the results on the ELISA testing using Rec-40 and *N*-terminal truncated acrosin proteins with mouse sera from GI strongly suggest the presence of immunoglobulins toward the carboxy protein region and support the notion that inhibition of ZPA interaction to Rec-40 could, at least in part, be attributed to immunoglobulins from the immunized mice reactive toward the proacrosin C-terminal protein end.

Antiacrosin antibodies developed using GI also were found to impair proacrosin activation, although inhibition of activity was not significant. In a report published elsewhere, a monoclonal antibody reactive to a conformational epitope of human acrosin was found to inhibit disaggregation of hamster ZP by the human enzyme but did not inhibit the hydrolysis of the substrate benzoylarginine ethyl ester by acrosin (82).

In vitro fertilization protocols performed to assess the effect of the antiacrosin antibodies upon sperm–oocyte interaction involved co-incubation of immunoglobulins toward proacrosin/acrosin with oocytes. This design was chosen considering findings elsewhere showing the inhibitory effect of anti-boar acrosin antibodies when added to the gamete incubation medium but not during sperm capacitation (83, 84). The results showing a significant inhibition of mouse IVF by the addition of antiacrosin antibodies raised by GI are in agreement with a report elsewhere using 630 μg/mL of a polyclonal antibody to a synthetic peptide of h-proacrosin (amino acids 43–60) (39). The mechanism of blockade by the antiacrosin antibodies was not characterized; other studies have reported the negative effect of antiacrosin antibodies upon sperm–oocyte binding (84), acrosomal exocytosis on the ZP matrix (Furlong LI, Liu Y, Baker HW, Veaute C, Vazquez-Levin MH, unpublished data) and penetration of zona-free hamster oocytes by bound acrosome-reacted sperm cells (85). The ability of these antibodies to block the interaction between ZPA and the human proenzyme would suggest that they may affect the interaction between the penetrating spermatozoa and the ZP (i.e., secondary binding). Recent studies reported the inhibition of IVF by specific antibodies toward IAM38, a protein localized to the inner acrosomal membrane with ZP-binding activity (19).

In addition to the deleterious effect upon fertilization, antibodies toward the human proacrosin/acrosin system had a negative impact upon early embryo development, cell viability, and embryo hatching. Reports elsewhere have shown the inhibitory effect of antisperm antibodies in sera from immunoinfertile patients toward human (86–89) and mouse (89–91) embryonic development; other studies described an inhibitory effect upon embryonic development after passive immunization with specific antisperm monoclonal antibodies YWK-1 (92) and ZAP-7 (93), as well as by in vitro development of two-cell embryo in the presence of monoclonal antibodies to specific sperm proteins (i.e., FA-1 [94]). Further studies will be required to determine whether an antigenic cross-reactivity with other embryo proteinases is responsible for the deleterious effect of the antiacrosin antibodies.

Animal immunization with the plasmid encoding h-proacrosin did not produce a significant inhibition of the number of pups, although a trend toward a decrease was observed, and their average size was significantly smaller than in the control group. Considering that the addition of antiacrosin antibodies significantly inhibited IVF and EED, it could be speculated that there may have been an insufficient amount of antibodies accessible at the site of fertilization to effectively block in vivo gamete interaction; modifications in the immunization protocol could be tested to increase the titers of circulating antibodies. Nevertheless, the lack of significant inhibition in the number of pups could be explained by the findings from the knockout studies that proved the ability of mouse spermatozoa to achieve fertilization in the absence of the proacrosin/acrosin system (95, 96).

In conclusion, the results here presented have shown that mouse GI with the sequence encoding h-proacrosin induces the production of antibodies in female mice; antibodies were capable of recognizing the human and mouse proacrosin/acrosin system in whole cells and protein extracts, significantly inhibited mouse IVF and EED, and showed a trend toward the impairment of in vivo fertility.

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