# Acrosin antibodies and infertility. I. Detection of antibodies towards proacrosin/acrosin in women consulting for infertility and evaluation of their effects upon the sperm protease activities

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**Objective:** To detect the presence of antibodies to the proacrosin/acrosin system and to evaluate their effect on the sperm acrosomal protein activities in women consulting for infertility.
**Design:** Retrospective study.
**Setting:** Basic research laboratory.
**Patient(s):** Recombinant proteins derived from human proacrosin (Rec-40, Rec-30, Rec-20, Rec-10) and recombinant human zona pellucida (ZP) glycoprotein A* (rec-hZPA).
**Intervention(s):** Development of an ELISA-Acro to test for antiacrosin antibodies using Rec-40 and truncated acrosin proteins as antigens.
**Main outcome measure(s):** Evaluation of: 1) the presence of antiacrosin antibodies; 2) the protein regions recognized by the antibodies; 3) the relationship between antiacrosin antibodies and surface antisperm antibodies (ASA) identified by the immunobead binding test (IBT); and 4) the effect of antiacrosin antibodies upon proacrosin/acrosin binding activity to ZPA and acrosin amidase activity.
**Result(s):** Antiacrosin antibodies were detected in sera from 34 of 179 women (19%). Detection of ASA by the IBT resulted in a similar incidence (36 of 179, 20%), although only six of them showed correspondence between both assays; five of these six sera were IBT-positive IgGs to the sperm head. Antiacrosin antibodies directed toward different protein regions inhibited proacrosin binding activity to rec-hZPA as well as its activation and acrosin amidase activity in protein sperm extracts.
**Conclusion(s):** Antiacrosin antibodies are present in sera of women consulting for infertility in both IBT-positive and IBT-negative samples, and they affect proacrosin/acrosin activities. (Fertil Steril® 2009;91:1245–55. ©2009 by American Society for Reproductive Medicine.)

**Key Words:** Human, infertility, immunologic, female, women, spermatozoa, proacrosin, acrosin, antisperm antibodies, immunobead binding test, ELISA, ZP, binding, proteinase activity, amidase, recombinant proteins

Presence of antisperm antibodies (ASA) has been associated with male and with female infertility, although reports show a rather variable incidence, ranging from 2% up to 55% of cases (1–3). In women consulting for infertility, the presence of ASA has been detected in serum, follicular fluid, and cervical mucus (4–7), accounting for conception failure in numerous cases (8). The ASA have been found to differentially bind to capacitated sperm cells (9, 10) and to affect several gamete functions, such as survival and motility (11), acrosomal exocytosis (12–14), and gamete interaction (15–18); in addition, evidence suggesting a detrimental effect of ASA on early development of human and mouse embryos has been reported (19–21). Despite its relevance, the relationship between the presence of ASA and its effect on fertility has remained difficult to establish; this is, at least in part, due to variations in serum concentrations of antibodies in infertile patients (3) as well as to ASA complexity, most likely comprising a vast repertoire of antibodies interacting with multiple sperm components (3, 22, 23). Although in many cases an impairment of sperm functions has been demonstrated, not all ASA will alter them, because either the antigen is not directly involved in a sperm biologic process or the antibodies do not bind to a functional domain of the antigen, as previously suggested (23).

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Among the available tests to evaluate the presence of ASA in spermatozoa, in female and male reproductive tract fluids in serum, the immunobead binding test (IBT) has been extensively used worldwide. The IBT allows the detection of immunoglobulins directed to the sperm surface, and provides a rough identification of the sperm region to which antibodies are directed (sperm head, midpiece, and tail) for each immunoglobulin class tested (IgG, IgA, IgM) (24, 25). Investigations to identify antigens recognized by ASA have been made using one- and two-dimensional polyacrylamide gel electrophoresis (PAGE) coupled to Western immunoblotting with ASA (26–29); unfortunately, this approach can not be applied in routine studies.

Through the years, numerous groups have dedicated their efforts toward the identification of sperm proteins involved in fertilization (30–33). The development of recombinant DNA technologies allows the use of proteins produced in heterologous systems (i.e., bacteria) to design immunochemical tests and experimental models to search for the presence and effects of antibodies directed to a specific antigen of interest. The identification of antibodies toward specific sperm proteins known to participate in the mechanism of sperm-egg interaction and the assessment of their effect in experimental models will hopefully allow a better understanding of the mechanisms behind immunologic infertility as well as improve the diagnosis and treatment of this reproductive pathology.

Acrosin (EC 3.4.21.10) is a serine protease found in the acrosome of spermatozoa of all mammalian species studied (34), as well as chicken (35) and ascidian (36). It is synthesized and stored as a single-chain zymogen, proacrosin; during the acrosome reaction, proacrosin is converted to the mature enzyme beta-acrosin and released from the acrosome (37, 38). The mechanism of activation toward the active enzyme is thought to involve processing of the proenzyme C- and N-terminal regions (39, 40). Evidence has accumulated supporting the participation of the proacrosin/acrosin system in eutherian fertilization: the enzymatic activity of acrosin is involved in the dissolution of the acrosomal matrix and the regulated release of the acrosomal contents during the acrosomal reaction (41). In addition, it was previously suggested that proacrosin/acrosin would participate in sperm–zona pellucida (ZP) binding by interaction with ZP glycoproteins (36, 42–45), and the mature enzyme would help sperm penetration by limited and specific proteolysis of ZP glycoproteins (34), although the latter role is currently under revision (46).

Specific antibodies to human proacrosin/acrosin have been developed in animal models (44, 47, 48), demonstrating the ability of this protein system to trigger an immune response. Moreover, in vivo (49, 50) and in vitro (51, 52) studies have demonstrated that sperm-ZP binding and enzymatic activities may be inhibited by specific antiacrosin antibodies. Despite this evidence, little is known about the incidence of circulating antiacrosin antibodies in humans and their potential effect on protein functions.

The aim of the present study was to determine whether antiacrosin antibodies were present in sera of women consulting for infertility, and to evaluate the effect of these antibodies upon protein activities.

In the accompanying paper, "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" (53), a genetic immunization model generated in mice by injection of a plasmid containing the sequence encoding h-proacrosin, and evaluation of the effect of antiacrosin antibodies on the proacrosin/acrosin activities and its impact on in vitro fertilization, early embyonic development and animal fertility is described.

## MATERIALS AND METHODS

### Chemicals

Unless specified, chemicals were of analytic 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).

### Sera and Spermatozoa

Studies done with human samples (women sera and semen) were approved by the Committee on Research in Human Subjects of the State University of New York and by the Ethical Committee at the Instituto de Biología y Medicina Experimental.

Serum samples were obtained from 179 women who varied in age from 21 to 43 years presenting at the Department of Obstetrics and Gynecology, Stony Brook University Medical Center, with either primary or secondary infertility. For women $<35$ years old, a conception delay of $\geq 12$ months was required before initiation of an evaluation, unless there was a past history of irregular menses suggesting an ovulatory disorder, or pelvic inflammation suggesting possible tubal disease. In women $>35$ years old, an evaluation was begun in the absence of conception after 6 months of unprotected coitus.

Sera were submitted for the IBT in preparation for intrauterine insemination (IUI) in cases of abnormal postcoital tests (failure of spermatozoa to enter preovulatory cervical mucus or their impaired motion within) or unexplained infertility. These couples had a semen analysis and a hysterosalpingogram to confirm tubal patency before IUI as part of our protocol. All women had a complete medical history and physical exam to exclude other illnesses that might contribute to their infertility.

To establish the cut-off value of the ELISA-Acro test, a total of 142 control serum samples were used (control group). These samples were obtained from a group of healthy women attending a clinical laboratory for routine blood testing that

included complete blood count, hematocrit, and erythrocyte sedimentation rate. The age range of the control women was 20–44 years. Patients were randomly selected by saving an aliquot of every other serum sample collected until reaching the number reported in the study; no data regarding their fertility status was available, although the result of testing a subpopulation of these samples (n = 30) by IBT revealed less than 5% frequency of ASA.

Serum immunoglobulins were obtained by 33%–66% ammonium sulphate fractionation from whole patient sera; the precipitated protein was dissolved in distilled water, and salt was removed by gel filtration using Sephadex G-25 (1,000–5,000 kDa).

Semen samples were obtained from normozoospermic donors according to World Health Organization standards (24). Only samples with more than 90% live spermatozoa, 75% progressive motile cells, and over 14% normal sperm forms (Kruger strict criteria) were included in the study.

### Detection of Surface ASA in Patients' Sera

The indirect IBT was performed according to Bronson and Tung (25). Briefly, a sperm suspension free of seminal plasma was obtained by Isolate (Irvine Scientific, Santa Ana, CA) density gradient (90:70:40) centrifugation of semen samples from normal donors known to have undetectable levels of ASA. Highly motile spermatozoa were recovered and incubated for 1 h at 37°C with a 1:10 dilution of the patient's serum in Dulbecco's phosphate-buffered saline (PBS, pH 7.4; Gibco, Grand Island, NY) supplemented with 5 mg/mL of bovine serum albumin (BSA; A7906; Sigma). At the end of the incubation, sperm cells were washed in the same buffer and resuspended in 50 μL PBS supplemented with 5 mg/mL BSA plus 10 μL of heat-inactivated horse sera (H1138; Sigma). Ten to fifteen microliters of the cell suspension was added to 50 μL of each prewashed isotype-specific immunobead set (IgG, IgM, and IgA) in 12 × 75 mm test tubes, gently mixed, transferred to a glass slide and coverslip, incubated 1–2 min, and sperm-bead binding scored. The number of motile spermatozoa with bound immunobeads, as well as bead localization (sperm head, midpiece, or tail, or combination of different regions: head and midpiece, head and tail, midpiece and tail, or head with midpiece and tail) was evaluated using a Nikon microscope with phase-contrast microscopy at ×400 magnification. Two hundred motile sperm cells were counted, scoring only those cells making contact with beads. At least 25% of sperm observed with bound immunobeads was considered to be positive.

### Production and Purification of Recombinant Human Acrosin Proteins

Recombinant human proacrosin (Rec-40) and N-terminal fragments (Rec-30, Rec-20, and Rec-10) were obtained using the pET-22b prokaryotic expression vector as previously described (44). Briefly, *Escherichia coli* BL21 (DE3) cells were transformed with a plasmid containing the cDNA sequence encoding human proacrosin (pET-525 clone). Bacteria were cultured in Terrific broth medium (2.4% bactoyeast extract, 1.2% bactotryptone, 0.4% glycerol, 0.17 mol/L $KH_2PO_4$, and 0.072 mol/L $K_2HPO_4$) containing 50 μg/mL carbenicillin at 37°C until $A_{600}$ = 0.6–1, and expression was induced by the addition of 1 mmol/L isopropyl-thio-β-D-galactoside; cells were harvested 2 h after induction, concentrated by centrifugation, and pellets kept at −70°C until used.

To recover the recombinant proteins, cell pellets were resuspended in a 1:10 culture volume of lysis buffer (50 mmol/L Tris-HCl, pH 8.0, 2 mmol/L EDTA, 1% Triton X-100, 1% sodium deoxycholate, and a cocktail of protease inhibitors) supplemented with 1 mol/L urea. The suspension was sonicated 3 times for 30 s at 30% of maximal power (Sonifier Cell Disruptor, model W140; Heat Systems–Ultrasonic, Plainview, NY). The insoluble fraction obtained after cell centrifugation (15 min at 10,000*g*) was resuspended in the same lysis buffer supplemented with 4 mol/L urea and sonicated and centrifuged again; the pellet was dissolved in Laemmli sample buffer (54) and subjected to preparative 12% sodium dodecyl sulfate (SDS) PAGE. Gel regions of interest were excised, and proteins were passively eluted in 0.1 mol/L Tris-HCl (pH 8.0), containing 0.1% SDS. Proteins were precipitated using the method previously described (55); briefly, 0.1 mL sample was added to 0.4 mL methanol, 0.1 mL of chloroform, and 0.3 mL of distilled water, mixed, and centrifuged at 9,000*g* for 1 min. The upper phase was removed and 0.3 mL methanol was added, mixed, and centrifuged at 9,000*g* for 2 min. Pelleted proteins were resuspended in PBS containing 4 mol/L urea, and protein concentration was determined (56) using BSA as standard.

### Production and Purification of Recombinant Human ZPA

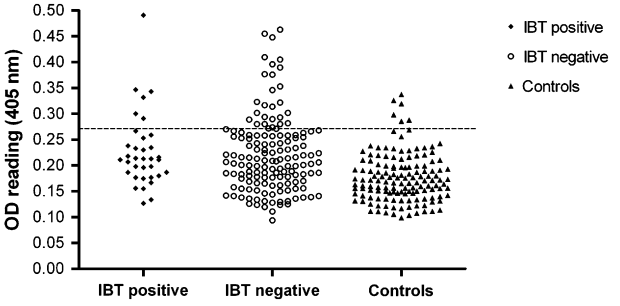
Recombinant human ZP glycoprotein A (rec-hZPA) was expressed and purified from Chinese ovary hamster cells as previously described (57); its average molecular mass is 105 kDa.

### ELISA for Detection of Anti–proacrosin/acrosin Antibodies

The presence of antibodies to the proacrosin/acrosin system in patients' sera was evaluated by an ELISA developed for the study (ELISA-Acro) using purified Rec-40, Rec-30, Rec-20, and Rec-10 acrosin proteins as antigens. Adequate amounts of the immobilized antigen, as well as serum dilutions and conjugate concentrations, were determined by checkerboard titration. Polystyrene microplates (Costar, Corning, NY) with 0.7 μg of recombinant acrosin proteins diluted in PBS supplemented with 4 mol/L urea per well were placed at 37°C for 2 h followed by overnight incubation at 4°C. Microplate wells were washed three times with PBS containing 0.05% Tween-20 (PBS-T). To block nonspecific binding sites, wells were incubated with PBS and 1% BSA at 37°C for 2 h and washed three times with PBS-T. Patients' sera were added to the wells in a 1:50 dilution (PBS-T with 1% BSA), incubated at 37°C for 1 h, and washed. Presence of antibodies was detected using

**FIGURE 1**

Reactivity of immunoglobulins in infertile (n = 179) immunobead binding test (IBT)–positive (solid diamonds) and –negative (open circles) and control (n = 142; solid triangles) patients' sera tested in the ELISA-Acro test with recombinant h-proacrosin (Rec-40) as screening antigen. Thirty-four sera (19%) from infertile patients showed a positive reaction, with 6 IBT positive and 28 IBT negative. In the control group, 6 sera (4%) were positive. Dotted line indicates cut-off value at 0.27 optical density units (OD).

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a system containing biotinylated polyvalent antihuman immunoglobulins diluted 1:5,000 in blocking solution and incubated for 30 min at 37°C; at the end of the incubation, wells were washed with Tris-buffered saline containing 0.05% Tween-20 (TBS-T) and then incubated at 37°C for 30 min with a 1:10,000 dilution of extravidin–alkaline phosphatase conjugate in TBS-T with 1% BSA. After washing with TBS-T, disodium *p*-nitrophenyl phosphate (*p*-NPP; 0.5 mg/mL in 0.1 mol/L diethanolamine, pH 9.8, with 0.5 mmol/L $MgCl_2$) was added as substrate and incubated at 37°C for 30 min. Readings were done at 405 nm in a Beckman Coulter microplate reader (Emax Precision Microplate Reader; Fullerton, CA). Results were expressed as absorbance values in optical density units (OD). A positive control with monoclonal antibody AcrC5F10 (Biosonda; Santiago, Chile) and biotinylated goat antimouse IgG was included in all runs. The absorbance cut-off value of the assay was established at 0.270 OD by calculating the mean absorbance value plus 2 SD of the readings obtained in 142 samples from the patients undergoing routine clinical evaluation (control group; Fig. 1).

### Proacrosin-ZPA Binding

Binding of proacrosin to rec-hZPA was assessed as recently described (45). Polystyrene plates were coated with 5 pmol/well of Rec-40 and incubated with rec-hZPA (1.0 μg/well). Binding was detected using an anti-hZPA rabbit serum (1:1,000 dilution). Then wells were incubated with biotinylated antirabbit IgG (1:500 dilution) and extravidin–alkaline phosphatase. Color reaction was developed using *p*-NPP, as already described. To evaluate the effect of antiacrosin antibodies on proacrosin-ZPA binding, plates coated with Rec-40 were incubated with immunoglobulins before adding hZPA. The rate of inhibition was determined by calculating $[1 - (OD_{Ig}/OD_0)] \times 100$, where $OD_{Ig}$ is the OD obtained in the presence of immunoglobulin and $OD_0$ is the OD obtained in the absence of immunoglobulin.

### Assessment of Acrosin Enzymatic Activity

Acid sperm extracts were prepared essentially as previously described (40). Briefly, semen samples were washed twice in PBS supplemented with 50 mmol/L benzamidine and 2 mmol/L *p*-aminobenzamidine to eliminate seminal plasma components. Sperm were resuspended in 1 mmol/L HCl with 50 mmol/L benzamidine and 10% glycerol, sonicated 3 times for 1 min at 25%–30% of power, placed for 18 h at 4°C, and centrifuged at 12,000*g*; the supernatant was dialyzed against 1 mmol/L HCl for 2 days to remove the protease inhibitors. Total protein content was determined by the Lowry et al. method (56).

Five micrograms of protein extracts were incubated at 37°C in triethanolamine buffer (pH 7.8) to induce activation of sperm proenzymes. Assessment of acrosin amidase activity was performed using 2 mmol/L N-benzoyl-DL-arginine *p*-nitroanilide (DL-BAPNA) as substrate in ELISA microtiter plates (Costar). The reaction was followed for up to 2 h to monitor the development of enzymatic activity every 30 min. Amidase activity was determined spectrophotometrically at 405 nm in an ELISA reader. An aliquot of the sperm extract supplemented with 100 mmol/L benzamidine to inhibit protease activity was included in all runs (negative control). The effect of antiacrosin antibodies on proenzyme activation and amidase activity was evaluated by adding 0.6 μg of partially purified immunoglobulins (see preceding) into the reaction mixture before or after zymogen activation, respectively, to a final 300 μL reaction volume. Evaluations were performed in duplicate in protein extracts from semen samples of at least two different donors. The rate of inhibition was determined as already described, considering as 100% of the activities estimated on readings obtained at 120 min incubation.

### Statistical Analysis

All experiments were repeated at least three times, and duplicate measurements were done. Data were expressed as mean ± SD. Statistical data analysis was performed using Statistica for Windows version 5.0 (Statsoft, Tulsa, OK). Data was analyzed, when appropriate, with chi-squared test, Student *t* test, and analysis of variance followed by multiple comparison when significant differences between mean values was found; alternatively, nonparametric tests (Mann-Whitney) were applied. Differences were considered to be significant when $P<.05$.

## RESULTS

### Detection of antiacrosin antibodies in sera from women consulting for infertility and relationship to surface antisperm antibodies

The presence of surface ASA was assessed with the IBT using a standard procedure in 179 sera from women consulting for infertility. As a result of the evaluation, 36 samples (overall incidence of 20%) were found to be IBT positive for IgG and IgA isotypes (>50% spermatozoa with bound immunobeads). In 15 cases, IgG isotypes mainly directed toward sperm head antigens (60%–99% spermatozoa with bound beads) were identified, and in 2 of the 15 cases a strong reactivity was observed to the sperm tail (97% and 99%). Immunoglobulin A isotypes to sperm head antigens were found in 13 positive sera (53%–97% spermatozoa with bound beads), two of which also had high percentage of IgG bound. Ten positive sera contained antibodies reactive mainly to the sperm tail (8 IgG, 1 IgA, and 1 IgG·IgA).

To detect the presence of antiacrosin antibodies, an ELISA was designed using recombinant human proacrosin (Rec-40, residues 1–402) as antigen (ELISA-Acro). As described in Materials and Methods, the cut-off value of the assay was established at 0.270 OD (Fig. 1). Based on this calculation, six samples from the control group showed levels of antiacrosin antibodies above the baseline value (4% incidence). A similar evaluation done in the group of patients undergoing infertility checkup revealed the presence of antiacrosin antibodies in 34 of the 179 cases (19%), which was significantly higher than the incidence found in the control group ($P<.0001$; chi-squared test). Detection of ASA by the IBT and of antiacrosin antibodies by the ELISA-Acro test rendered a similar percentage of affected cases; however, only six of them showed correspondence in the results between both assays, and five of these six sera were IBT-positive IgGs to the sperm head (Table 1).

Proacrosin regions recognized by the antibodies were evaluated by performing the ELISA-Acro assay using recombinant N-terminal fragments of h-proacrosin produced in bacteria as antigens. The truncated proteins comprise amino acids 1–300 (Rec-30), 1–190 (Rec-20), and 1–160 (Rec-10) of proacrosin, as previously reported (44) (Fig. 2). Of the 34 immunoreactive sera to Rec-40, 25 were found to react with Rec-30, indicating that 9 sera carried antibodies recognizing only sequences between residues 300 and 402 of h-proacrosin; moreover, antibodies to Rec-20 and Rec-10 were detected in 9 of the 34 sera reacting with Rec-40. Therefore, 73% (25 of 34) of the positive sera to h-proacrosin had antibodies that recognize epitopes localized between residues 1 and 300 (9 to amino acids 1–190 and 16 to residues 190–300), and a group of 9 sera (27%) were specific to the C-terminal region of h-proacrosin (Fig. 2: green bars). These results revealed the presence of antiacrosin antibodies along several regions of the proenzyme. These findings are in agreement with a theoretic analysis of protein antigenicity (58) performed with the human proacrosin sequence, which revealed the presence of antigenic determinants along the proenzyme sequence (data not shown).

**TABLE 1**

**Antisperm antibody binding in immunobead binding test (IBT) and antiacrosin antibody reactivity in ELISA of sera from infertile patients giving positive results by both tests.**

| | Percentage binding in IBT (head/tail) | | | |
|---|---|---|---|---|
| Sample no. | IgG | IgA | IgM | ELISA (OD) |
| AI395 | 72[a]/10 | 4/12 | 15/35 | 0.492 |
| AI967 | 27/87 | 0/17 | 4/15 | 0.348 |
| BZ029 | 65[a]/15 | 0/1 | 1/8 | 0.300 |
| BZ099 | 72[a]/22 | 0/3 | 2/60 | 0.310 |
| BZ801 | 69[a]/20 | 0/3 | 1/2 | 0.343 |
| BA093 | 76[a]/35 | 0/11 | 6/39 | 0.333 |

*Note:* OD = optical density units.
[a] Positive IgG to the sperm head.

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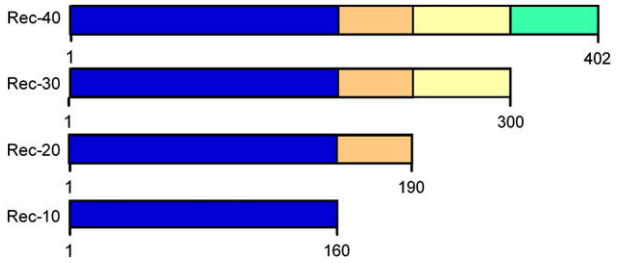
Altogether, these studies showed an incidence of antibodies to the proacrosin/acrosin system, directed to different regions of the protein, in 19% of cases consulting for infertility. The presence of these antibodies could not be anticipated by the IBT in many of cases, revealing a low rate of correspondence between the results of the IBT and those of the ELISA-Acro assessment.

### Effect of Antibodies on Proacrosin/Acrosin Activities

**Proacrosin binding activity toward ZPA** Studies done by our group in the human model have shown that proacrosin/acrosin proteins bind to ZP glycoproteins (44), and demonstrated

**FIGURE 2**

Schematic representation of bacterial recombinant human proacrosin/acrosin proteins: Rec-40 (residues 1–402; blue + orange + yellow + green bars), N-terminal fragments Rec-30 (1–300; blue + orange + yellow bars), Rec-20 (1–190; blue + orange bars), and Rec-10 (1–160; blue bar). The green bar represents the C-terminal region.

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that the recombinant proenzyme Rec-40 had the highest binding affinity to rec-ZPA (Kd 34 nmol/L), the mouse ZP2 homologue, suggesting a relevant role for the proenzyme C-terminal region in the binding process (45). Therefore, it was of interest to evaluate the effect of the antibodies directed to the C-terminal region of proacrosin on such interaction in a subgroup of patients' sera. Proacrosin–rec-hZPA binding was evaluated in the presence of the serum immunoglobulins from the nine sera recognizing the C-terminal region of Rec-40. Immunoglobulins from these positive sera, as well as from antiacrosin-negative sera were added at four different concentrations (500, 100, 10, and 1 $\mu$g/mL). As shown in Figure 3A, a trend toward a decrease in binding of proacrosin to rec-hZPA was observed when the assay was performed in the presence of the antiacrosin-positive sera; when added at 1 $\mu$g/mL, a significant inhibition in positive sera was observed, ranging from 30% to 49% inhibition (median value 42%; $P<.001$; Mann-Whitney test), whereas those from negative sera inhibited between 6% and 15% (median value 12%); similar results were obtained when immunoglobulis were added at 10 $\mu$g/mL (32%–50% vs. 0%–21%; $P<0.001$; Mann Whitney test). All tested sera directed toward the C-terminal region of proacrosin had an inhibitory effect on interaction of the proenzyme with rec-hZPA, with a minimum inhibitory effect of 32% when tested at 10 $\mu$g/mL; immunoglobulins from negative sera showed a maximum blocking effect of 21% when added at the same concentration (Fig. 3B).

**Proacrosin activation and acrosin activity** Conversion of proacrosin into its enzymatically active form, $\beta$-acrosin, involves protein processing at the C-terminal portion of the proenzyme (39, 40); this process can be achieved in vitro by raising the pH of protein sperm acid extracts to 7.8 (40). To evaluate the effect of antiacrosin antibodies on h-proacrosin activation, immunoglobulins obtained from antiRec-40–positive sera were added at the beginning of the proenzyme activation process, as described in Materials and Methods. Under the conditions assayed, h-proacrosin activation diminished (>20%) in three of the nine sera containing antibodies that recognized the C-terminal protein region, and in one of the three cases, the inhibition was significant (46 $\pm$ 3% of inhibition; $P<0.05$; Mann-Whitney test; Table 2A).

The effect of immunoglobulins on acrosin amidase activity from the 25 sera reacting to the recombinant acrosin protein Rec-30 (the sequence spanning $\beta$-acrosin) was also evaluated. When the assay was performed under standard conditions (5 $\mu$g protein and 2 mmol/L DL-BAPNA), a discrete decrease in the enzymatic activity was observed in four sera containing antiacrosin antibodies (16%, 19%, 19%, and 23%; Table 2B); when the enzyme/subtrate ratio was changed to 2 $\mu$g of protein sperm extract and 0.13 mmol/L DL-BAPNA, two out of four samples tested showed a significant decrease in acrosin activity (63 $\pm$ 2% and 62 $\pm$ 1% of inhibition at 120 min vs. control $P<0.05$; Mann-Whitney test).

Altogether, these studies showed that antiacrosin antibodies directed to the C-terminal region of the proenzyme are capable of inhibiting proacrosin ability to interact with rec-hZPA as well as its activation to the mature active form. Moreover, in some cases, proteinase activity was negatively affected when antiacrosin antibodies directed to epitopes located in the polypeptide sequence of the mature enzyme were added to the activity assay.

## FIGURE 3

Inhibition of proacrosin-ZPA binding by antiacrosin antibodies. Rec-40 was immobilized on microtiter plates, and binding to rec-hZPA was assessed as described in Materials and Methods. Purified immunoglobulins (500, 100, 10 and 1 $\mu$g/mL) from patient and control sera were added, and the rate of inhibition of binding was determined. (**A**) Rate of inhibition of Rec-40 binding in positive (*green*) and negative (*red*) sera; results are median values and 25th and 75th percentiles (*bars*). *$P<.001$ vs. negative sera (Mann-Whitney test). (**B**) Adding 10 $\mu$g/mL antibodies from patient sera produced 32%–50% inhibition, whereas antibodies from control sera produced a maximum of 21% inhibition. Points above the horizontal dotted line are ELISA-positive sera (optical density units [OD] >0.27). Vertical dotted lines show inhibition of binding of 21% and 32%.

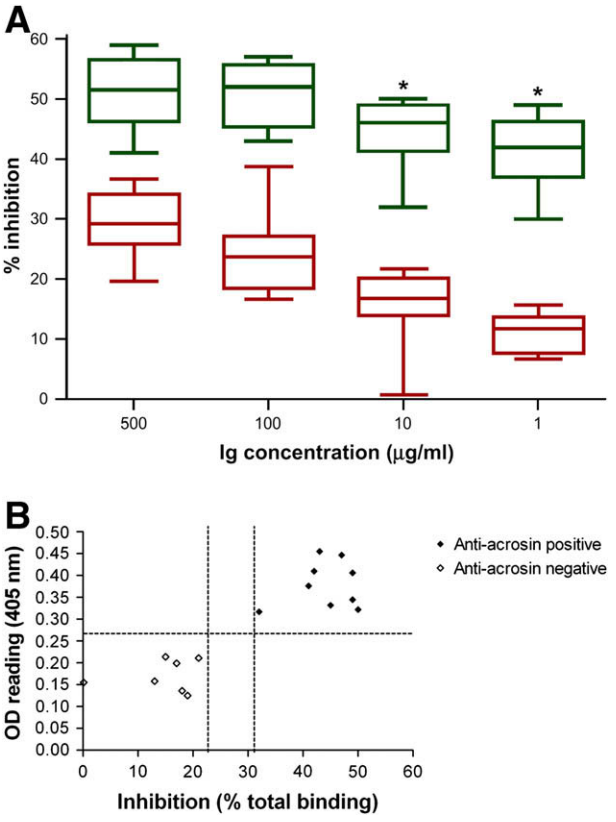


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**TABLE 2**

**Inhibition of proacrosin activation and activity by immunoglobulins from sera recognizing only Rec-40. Amidase activity was determined on human sperm protein extracts using DL-BAPNA as substrate, and 0.6 μg of purified immunoglobulins from individual patients' sera were added at the beginning (A) or after (B) the activation period. Inhibition rates were calculated using optical density measures at 120 min of reaction.**

| | Serum | Inhibition of proenzyme activation (% decreased activity)[a] |
|---|---|---|
| (A) AntiRec40-positive sera | AI329 | 23 |
| | AI363 | 5 |
| | AI558 | 46[b] |
| | AI905 | 6 |
| | AI922 | 24 |
| | AI974 | 14 |
| | BZ083 | 18 |
| | BA093 | 15 |
| | BA653 | 0 |
| (A) AntiRec40-negative sera | AF735 | 4 |
| | AF544 | 8 |
| | AF668 | 12 |
| | AI446 | 19 |
| | BZ708 | 6 |
| | AI536 | 9 |
| | AI338 | 6 |
| (B) AntiRec30-positive sera | AI332 | 12 |
| | AI340 | 11 |
| | AI395 | 16 |
| | AI458 | 3 |
| | AI469 | 3 |
| | AI536 | 0 |
| | AI634 | 23 |
| | AI872 | 2 |
| | AI967 | 19 |
| | BZ008 | 2 |
| | BZ018 | 8 |
| | BZ029 | 0 |
| | BZ078 | 8 |
| | BZ215 | 9 |
| | BZ708 | 7 |
| | BZ794 | 15 |
| | BZ801 | 9 |
| | BZ843 | 13 |
| | BZ960 | 8 |
| | BA121 | 0 |
| | BA171 | 12 |
| | BA223 | 0 |
| | BA355 | 6 |
| | BA376 | 9 |
| | BA445 | 19 |
| | BA467 | 0 |
| (B) AntiRec30-negative sera | AF544 | 5 |
| | AF668 | 6 |
| | AI446 | 5 |
| | BZ708 | 8 |
| | AI536 | 9 |
| | AI338 | 2 |
| | AF735 | 3 |

*Note:* DL-BAPNA = N-benzoyl-DL-arginine *p*-nitroanilide.

[a] Activity is expressed as percentage decrease from control without antibodies.

[b] $P < .05$.

*Veaute. Antiacrosin antibodies in subfertile women. Fertil Steril 2009.*

## DISCUSSION

Numerous reports have documented the involvement of the proacrosin/acrosin system in mammalian fertilization in humans as well as in animal models (41–45), and there are some studies that have demonstrated the ability of proacrosin/acrosin to trigger an immune response (44, 47–50). On the other hand, several publications have described the ability of circulating ASA to block fertilization by affecting sperm protein functions involved in this process (9–21). However, investigations on the detection of antiacrosin antibodies in patients consulting for infertility and the effect of these antibodies on the proacrosin/acrosin system activities have been rather elusive. In this study, an ELISA approach was developed to identify the presence of antiacrosin antibodies in patients, and evaluations were done to determine the effect of the immunoglobulin fraction from the reactive sera on the proacrosin/acrosin system activities.

Routine methods developed toward the assessment of the presence and localization of ASA, such as the IBT and the mixed agglutination reaction test, detect immunoglobulins directed mainly to sperm plasma membrane antigens (24). Those assays, however, do not evaluate the presence of immunoglobulins to other components that are of relevance to sperm physiology, e.g., the acrosome. Several acrosomal proteins, such as the proacrosin/acrosin system, are exposed in the site of fertilization when the sperm undergo acrosomal exocytosis; because these proteins have been shown to participate in sperm-egg interaction, presence of antibodies to these entities should be taken into consideration for their potential effect on sperm fertilizing ability.

To evaluate the presence of proacrosin/acrosin antibodies in sera from women consulting for infertility, an ELISA immunoassay (ELISA-Acro) was developed using proacrosin and acrosin fragments. ELISA assays have been extensively used in the detection of antibodies to sperm antigens using complex protein extracts (59–62) as well as to specific proteins as antigens (63–65), showing them to be effective in the detection of ASA as well as in quantitation of the signal, in addition to their simplicity, speed, and sensitivity.

In the ELISA-Acro, recombinant proacrosin (Rec-40) and carboxy terminus–truncated products (Rec-30, Rec-20, and Rec-10) produced by recombinant DNA technology in bacterial cultures and purified using preparative electrophoresis (44) were used as antigens. In addition to being produced in large quantities, purified bacterial acrosin proteins are free from other sperm polypeptides that could be copurified with the acrosomal enzyme from cell extracts and may interfere by reacting to other immunoglobulins present in the patients' sera. Assay optimization involved selection of adequate antigen concentration, immobilization buffer, and nonspecific blocking medium. Moreover, Rec-40 and truncated acrosin forms Rec-30, Rec-20, and Rec-10 were used to identify immunoreactive sera, allowing a partial epitope mapping analysis; considering that recombinant proteins are either partial or totally denatured, lack of detection of conformational epitopes can not be disregarded. To establish the ELISA-Acro cut-off value, sera from patients undergoing routine clinical blood testing were used. The formula used to calculate this value (mean OD obtained for negative sera + 2 SDM) expresses the upper limit of the 95% confidence interval for the negative sera distribution frequency, giving a 5% chance for a result to be above 0.270 on a test value from a healthy subject serum (66). The use of this calculation to determine a cut-off value has been previously reported (67, 68).

The results of the ELISA-Acro performed in sera from a subgroup of women undergoing infertility workup revealed an incidence of 19% of patients possessing antiacrosin antibodies above the baseline. In contrast, an incidence of 4% of patients with high levels of antiacrosin antibodies was established in the control group, a frequency significantly lower than in the study group, even though subjects were randomly selected without any exclusion based on fertility status.

The percentage of patients exhibiting antiacrosin antibodies determined in the study group was similar to that previously reported by Howe et al. (69). Despite the similarity between the results of both reports, a comparative analysis could not be done, because several differences were found between their designs. In the earlier study, the presence of antiacrosin antibodies was investigated only in ASA-positive sera assessed using ELISA and IBT and the incidence reported came from the evaluation of sera from a group of female and male patients; moreover, evaluations were done using Western immunoblot analysis with a sperm protein fraction enriched in $\beta$-acrosin but no further studies were done to determine the identity of the immunoreactive proteins to confirm that immunoglobulins were specific for acrosin proteins or other sperm proteins of similar molecular weight. Finally, Howe et al. did not report a quantitative analysis of the signal obtained for each analyzed serum.

Serum samples from the study group in the present study, a population of women undergoing IUI for treatment of infertility after abnormal postcoital tests or in the face of unexplained infertility, were subjected to a parallel analysis to assess the presence of surface ASA using the IBT. The incidence of ASA was similar to that previously reported in the literature, with a higher proportion of sera positive for the IgG class than for IgA class, as expected (4–7). The frequency of cases with antibodies detected by IBT was similar to that obtained for antiacrosin antibodies, but correspondence between results on samples evaluated by both methods was rather low, and the majority of patients' sera reactive to the proacrosin/acrosin proteins were negative in the IBT. The findings were not surprising, considering that these tests evaluate different targets (surface versus intracellular antigens); they stress, however, the concept that assessment of surface ASA is not an indicator for the presence of anti–proacrosin/acrosin antibodies, and possibly other intra-acrosomal proteins of functional relevance. In agreement with this reflection, our group previously described the presence of antisperm antibodies capable of triggering the acrosome reaction in follicular fluid samples which were negative by the IBT (14).

In the present study, evaluations were extended toward the identification of the proacrosin regions recognized by the patients' sera immunoglobulins. A higher incidence of samples with antibodies to the sequence encoding the active enzyme (n = 25) was observed in the acrosin-reactive samples compared with those only reactive to the protein carboxy terminus (n = 9). The use of ELISA in combination with truncated acrosin polypeptides is very helpful for these studies and provide information on protein antigenicity. Considering that protein sequences involved in proacrosin activation, proteinase activity, and ZP-binding activities are distributed along the entire molecule, these results prompted us to assess the effect on proacrosin/acrosin functions.

In agreement with earlier reports in boar (42), mouse (43) and ascidian (36), data from our group previously demonstrated that recombinat proacrosin (Rec-40) and N-terminal–truncated proteins have the ability to interact with native and recombinant ZP glycoproteins (44); further studies done by our group in the human model demonstrated that the recombinant proenzyme Rec-40 had the highest binding ability to rec-h ZPA, indicating an important role for the C-terminal sequences of the proenzyme (45). Interestingly, this protein region undergoes extensive processing during the proenzyme activation (39, 40). Moreover, acrosin has been characterized at the biochemical and molecular levels as a member of the trypsin family, and its mechanism of activation has been elucidated (39, 40). The effect of antiacrosin antibodies on both activities was tested by evaluating their ability to interfere in the interaction between the acrosomal enzyme and ZP glycoproteins as well as to affect the proenzyme activation/enzyme activity. Purified immunoglobulins from all patients' sera immunoreactive only to the proacrosin C-terminus were found to interfere with ZPA–Rec-40 binding, suggesting their association to motifs involved in the interaction. Human sperm protein extracts were used to assess the effect of antiacrosin antibodies on the proenzyme activation and enzymatic activity. Immunoglobulins from sera immunoreactive to the proacrosin carboxy-terminal end negatively affected proacrosin activation when added at the beginning of the activation process; in contrast, this subgroup of antiacrosin antibodies appeared not to affect acrosin amidase activity in sperm extracts previously incubated to promote proacrosin activation. On the other hand, immunoglobulins directed to the sequence encoding $\beta$-acrosin showed, in some cases, a significant negative effect on the acrosin amidase activity.

To our knowledge, this is the first detailed analysis evaluating the immune response to the human proacrosin/acrosin system in sera from women consulting for infertility. The results of this study show the presence and incidence of antiacrosin antibodies in sera of a selected group of women under infertility workup and a negligible incidence of these antibodies in serum samples from a random population. In addition to describing the presence of antiacrosin antibodies in female sera, the results of this analysis highlight the importance of assessing the immune response to sperm antigens other to those exposed at cell surface, e.g., intra-acrosomal proteins.

The studies here presented included the development of an ELISA to assess the incidence and levels of antibodies as well as a partial characterization of the antibodies' reactivity along the protein sequence. In addition, the studies showed a negative effect of the purified immunoglobulins from reactive sera on proacrosin activation, acrosin amidase activity, and proacrosin/acrosin ZP-binding activity. Because patient sera reactive to sperm antigens are rather complex, their effect on fertilization events could not be assessed with these tools. Complementary studies described in the accompanying paper (53) were aimed at the development of a murine model of immunity to human proacrosin achieved by genetic immunization, in which the effect of circulating antiacrosin antibodies on animal fertility, as well as on in vitro fertilization and early embryonic development, was evaluated.

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