Involvement of Fertilization Antigen (FA-1) in Involuntary Immunoinfertility in Humans

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Abstract

Sera from immunoinfertile patients (n = 32) and fertile controls (n = 20) were analyzed for cross-reaction with a purified and characterized sperm-specific glycoprotein, the fertilization antigen (FA-1), employing an enzyme-linked immunosorbent assay. The immunoinfertile sera demonstrated a strong reaction with FA-1 when compared with fertile control sera. There was no correlation between the reaction of sera with FA-1 and the titers obtained through the sperm agglutination technique and the sperm immobilization technique. Immunoinfertile sera showed binding with the protein bands in the regions corresponding to FA-1 on Western blots involving sodium deoxycholate–solubilized human sperm. Antigens isolated with immunoaffinity chromatography involving immunoinfertile sera also demonstrated antigen bands corresponding to FA-1 in sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Of the seven immunoinfertile couples, three that had antibodies to FA-1 in the male as well as female partners demonstrated a block of fertilization (IVF) due to antibodies bound on the sperm surface. The anti–FA-1 antibody activity was detected in serum as well as in follicular fluid and seminal plasma. Immunoinfertile sera that showed an inhibition of human sperm penetration of zona-free hamster ova showed a significant ($P < 0.001$) increase in penetration rates after absorption with FA-1. These results indicate that sera from immunoinfertile patients had antibodies reacting with FA-1, and these antibodies are involved in the fertilization process.

Introduction

Antispermatozoal antibodies have been implicated in involuntary infertility in humans (1–5). These antibodies inhibit human sperm penetration of zona-free hamster ova (6) and also reduce fertilization rates in in vitro fertilization–embryo transfer (IVF-ET)[1] procedure (7). The inhibition of fertilization rates may be due to interference in either the capacitation and acrosome reaction; zona binding and penetration and/or sperm-egg plasma membrane recognition and fusion. Antispermatozoal antibodies present in infertile sera are directed against numerous sperm antigens. Recently, using the Western blot procedure, attempts have been made to delineate antigens relevant to fertility (8). There are only a few sperm-specific antigens such as LDH-C_4, acrosin, and hyaluronidase that have been isolated in purified form and characterized. But these antigens rarely, if ever, react with the sera from immunoinfertile patients (9).

Recently, we isolated and characterized a sperm-specific glycoprotein, the fertilization antigen (FA-1), from human and murine male germ cell plasma membranes (10). The tissue-specific but species–cross-reactive monoclonal antibodies against FA-1 inhibit human sperm penetration of zona-free hamster ova and also block penetration of murine oocytes by murine sperm; they do so without causing agglutination or immobilization of sperm (11). The FA-1 also causes a reduction of fertility in actively immunized female rabbits by mechanism(s) involving an inhibition of the fertilization process (12). The present studies describe the presence of antibodies reacting with FA-1 in the sera of immunoinfertile patients. These studies further aim at obtaining relevant information regarding (*a*) the effects of the presence of anti–FA-1 antibodies on the outcome of IVF procedure, and (*b*) the effects of absorption of immunoinfertile sera with FA-1 on human sperm penetration of zona-free hamster oocytes.

Methods

Fertilization antigen (FA-1). FA-1 was purified from lithium diiodosalicylate (LIS)–solubilized murine testes by immunoaffinity chromatography involving monoclonal antibodies (10). Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), high-performance liquid chromatography (HPLC), density gradient ultracentrifugation, and amino acid analysis indicated that the purified antigenic molecule is composed of a monomer of 23,000 D and/or a dimer of 47,000–50,000 D (10). Each batch of FA-1 was tested for its homogeneity. Only those batches that showed specific bands of 23,000 and/or 47,000–50,000 D in sodium dodecyl sulfate (SDS)–PAGE when stained with ultrasensitive silver stain, were used in the present study.

Patient sera. Sera (n = 32) were collected from immunoinfertile patients, males and females (24–37 yr old), who demonstrated antisperm antibodies in their sera as revealed by the sperm agglutination technique (SAT) (13) and/or the sperm immobilization technique (SIT) (14). These sera will be referred to as immunoinfertile sera. 12 of these sera were collected from seven infertile couples admitted to our

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1. *Abbreviations used in this paper:* AAFS, affinity-purified antigens isolated with fertile serum; AAIS, affinity-purified antigens isolated with immunoinfertile serum; BWW, Biggers, Whitten, and Whittingham medium; DOC, sodium deoxycholate; FA-1, fertilization antigen; FCA, Freund's complete adjuvant; FCS, fetal cord serum; HSA, human serum albumin; IFA, incomplete Freund's adjuvant; IVF-ET, in vitro fertilization–embryo transfer; LIS, lithium diiodosalicylate; MS, maternal serum; SAT, sperm agglutination technique; SIT, sperm immobilization technique; SPA, sperm penetration assay.

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IVF-ET program for infertility treatment. Control sera (n = 20) were collected from fertile males and females (27–34 yr old), most of whom had sired a healthy baby or had been pregnant within the last year. Control sera did not demonstrate antisperm antibodies when tested by SAT and SIT. These sera will be referred to as fertile sera. Sera were heat-inactivated (56°C, 30 min) and stored at −20°C. Follicular fluids and seminal plasmas collected from the seven infertile couples undergoing IVF-ET procedure were also heat-inactivated (56°C, 30 min) and stored at −20°C.

Rabbit antisera against FA-1. Sexually mature virgin female rabbits of New Zealand white strain were injected systemically (12) at subcutaneous and intramuscular sites with either (*a*) a preparation of washed ejaculated human sperm membrane solubilized in 15 mM sodium deoxycholate (DOC) (12) or (*b*) purified FA-1 in Tris-HCl buffer (0.1 M, pH 8.0). The first injection consisted of 0.2 ml of solution containing 50–75 μg of the corresponding protein emulsified with an equal volume of Freund's complete adjuvant (FCA). 2 wk after the primary injection, the rabbits were injected weekly for 3 wk with 50 μg of the antigen mixed with incomplete Freund's adjuvant (IFA). 1 wk after the last injection, antisera were collected, heat inactivated (56°C, 30 min), and stored at −20°C. These antisera, namely the anti–DOC-human sperm (No. 102), anti–FA-1 (No. A, 3), and monoclonal antibodies raised against FA-1 (11) were used as positive controls.

Western blot enzyme immunobinding procedure. Antigenic specificities of the antisera and monoclonal antibodies were evaluated by the Western blot enzyme immunobinding procedure described earlier (15, 16). Briefly a ~ 1–2.5 mg protein solution of DOC (15 mM in 0.05 M Tris-HCl, pH 8.0)-solubilized washed ejaculated human sperm membrane preparation (pooled from three or more donors) was run under nonreducing conditions in the slab SDS-PAGE (10% polyacrylamide gel) according to the procedure described by Laemmli (17). Immediately after electrophoresis, the protein bands were electrophoretically transferred from the slab gel to nitrocellulose paper by the method of Towbin et al. (18). The reacted antigens were localized by incubating the strips of blotted nitrocellulose paper with sera (1:50) diluted in incubation buffer (50 mM Tris HCl, 150 mM NaCl, 5 mM EDTA, 0.25% gelatin, 0.05% NP-40, pH 7.4), goat anti-rabbit or goat anti-mouse or goat anti-human gamma immunoglobulins conjugated to peroxidase (1:800), and finally with the substrate solution (0.001% *p*-chloro-1-naphthol in 0.05 M Tris HCl, pH 7.4, containing 0.03% H_2O_2) as described earlier (15, 16).

Enzyme-linked immunosorbent assay (ELISA). ELISA was performed using antigen-coated discs (6-mm diameter, Cordis Laboratories Inc., Miami, FL). These discs are made of a poly-(tetrafluorethylene)-styrene graft copolymer substituted with isothiocyanate groups. ~ 3 μg of the purified FA-1, showing specific band(s) in SDS-PAGE, was dissolved in 5 ml of carbonate buffer (0.1 M, pH 9.6) and the solution was incubated with 20 discs overnight at 4°C. The discs were washed three times for 5 min each with phosphate-buffered saline (PBS, pH 7.4) containing 0.05% Tween 20 (PBS-Tween). To block the nonspecific binding sites, the discs were incubated with PBS-Tween plus 1% human serum albumin (HSA) at 37°C for 45 min and again washed three times with PBS-Tween. The discs were transferred individually into glass tubes and incubated with 1.0 ml of the serum (1:10 diluted in PBS-Tween + 0.5% HSA) at 37°C for 2 h. The discs were washed three times with PBS-Tween and incubated with 1.0 ml of goat anti-human immunoglobulin conjugated with alkaline phosphatase (1:1,500 diluted in PBS-Tween + 0.5% HSA) at 37°C for 1.5 h. The alkaline phosphatase conjugate (1:1,500 diluted) of goat anti-rabbit or anti-mouse immunoglobulin was used as the secondary antibody when using rabbit antiserum and hybridoma supernatant containing monoclonal antibodies, respectively. The discs were washed five times with PBS-Tween, transferred to new glass tubes, and incubated with 1.0 ml of the substrate solution (1 mg/ml solution of disodium *p*-nitrophenyl phosphate in 10% diethylanolamine buffer, pH 9.8) at 37°C for 30 min. The reaction was stopped by adding 1.0 ml solution of 3 N NaOH. Aliquots of 200 μl were transferred to the wells of a 96-well Dynatech microelisa plate and read at 405 nm.

Each serum was run in duplicates and the uncoated discs treated identically served as controls. The absorbance reading of the uncoated discs was subtracted from the absorbance reading of the antigen-coated discs and the mean of the subtracted values was recorded. The sera were run in batches of 10–15 and a known positive (No. 102 or A, or 3 appropriately diluted; aliquots were sampled and stored at −20°C) was included as a quality control in each batch. The absorbance readings were converted to the standard deviation (SD) units using the following formula: SD units = mean (test) − mean (control)/SD of control group. The test samples with SD units of ≧ +2 were considered as having a positive reaction with FA-1.

IVF procedure. The details of IVF procedure has been described elsewhere (19). A combination of clomiphene citrate (Serophene, Serono, Randolph, MA) and human menopausal gonadotropin (Pergonal, Serono) was used to enhance multiple follicle development, which was monitored by ultrasound and serum estradiol concentrations. Patients demonstrating no spontaneous luteinizing hormone surge were injected with human chorionic gonadotropin (10,000 U of hCG, Profasi HC; Serono). 34 h after the hCG injection, a laproscopic retrieval of the oocytes was performed as reported before (19). The oocytes were obtained from follicles in follicular fluid or follicular flush in Dulbecco's PBS. They were identified as 'mature' if they had fully expanded and stretchable homogeneous culumus (> 4 cm) with a radiating corona layer and a visible polar body, as 'intermediate' preovulatory oocytes if they had less stretchable granular appearing cumulus (< 2 cm) without a radiating corona layer, or as 'immature' if they had an intact germinal vesicle and had neither a cumulus nor a polar body. Within 2 min of retrieval, they were transferred to a preequilibrated Ham's F-10 medium containing 7.5% heat-inactivated (56°C, 30 min) fetal cord serum (FCS) pretested in a 72-h culture of two-cell murine embryos (20). Ham's F-10 (pH 7.69, 280 mosmol/kg) was prepared as described by Lopata et al. (21). A fresh semen sample was collected from the male partner and liquefied at 37°C for 30 min. The semen was analyzed as described earlier (22). Immediately after the liquefaction, the semen was overlayed with Ham's F-10 medium containing 7.5% FCS (8 ml). After 30 min at 37°C, the motile sperm in the overlay were centrifuged (500 *g*, 5 min), washed twice with Ham's F-10 medium containing 7.5% FCS, and 66,000 motile sperm were added to each dish having oocytes in 2 ml of the Ham's F-10 containing 7.5% FCS. Before insemination, oocytes were incubated (37°C, 5% CO_2 and 95% air mixture) for 5–9 h (mature), 10–23 h (intermediate), and 27–30 h (immature). They were examined 15 h after insemination for fertilization. They were defined as 'fertilized' if they had two pronuclei and a second polar body. The fertilization was further confirmed by the ability of the fertilized oocyte to cleave in the growth medium (Ham's F-10 containing 15% FCS). Spermatozoa were observed for any agglutination and loss of motility in the insemination medium. The zonae pellucidae were also checked for the sperm binding. The presence of anti–FA-1 antibodies were checked retrospectively and compared with the fertilization rates. The IVF performed in infertile patients who did not have antibodies to FA-1 served as controls.

Immunoaffinity chromatography. Immunoaffinity chromatography was performed as described elsewhere (10, 15). Immunoglobulins were separated from sera of an infertile man (No. 38) and a fertile man (No. 11) by stepwise precipitation with sodium sulfate (23). The immunoglobulins were coupled to Sepharose-4B activated by CNBr, and the coupled gels were packed into a column and equilibrated with 0.1 M phosphate buffer (pH 8.0). Washed ejaculated sperm from the fertile donor (No. 11) were solubilized in 0.3 M LIS in 0.05 M Tris-HCl (pH 8.0), containing 1 mM phenylmethylsulfonylfluoride (PMSF) and 5 mM soybean trypsin inhibitor at room temperature for 30 min, then at 4°C for 2 h and then centrifuged at 25,000 *g* for 30 min. (10). The LIS-solubilized sperm supernatant was dialyzed against 0.15 M lithium chloride and then against 0.05 M Tris-HCl (pH 8.0) containing 0.15 M NaCl and 1 mM EDTA to remove the chaotropic agent. The dialyzed antigen preparation (1 ml containing 3–5 mg of protein) was incubated with the immunoaffinity column for 3 h at room tempera-

ture or overnight at 4°C. The unbound proteins were washed at pH 8 (0.1 M phosphate buffer) and then at pH 4 (0.1 M glycine-HCl buffer) and the tightly bound proteins were eluted at pH 2.8 (0.1 M glycine-HCl buffer). The fraction eluted at pH 2.8 was immediately neutralized with 1 M KH_2PO_4, concentrated and dialyzed overnight against Tris-HCl buffer (0.1 M, pH 8.0), and checked in SDS-PAGE.

Sperm penetration assay and immunoabsorption with FA-1. The sperm penetration assay (SPA) was preformed as described by Yanagimachi et al. (24). Superovulation was induced in adult female golden hamsters by injecting with 25 IU pregnant mare serum gonadotropin (Sigma Chemical Co., St. Louis, MO) on the morning of the first day of the estrus cycle, followed 50 h later by injecting with 40 IU of hCG. The animals were killed 16–18 h after the hCG injection and the mature unfertilized ova were collected from the oviducts. The ova were separated from the surrounding cumulus cells by incubation in 0.1% hyaluronidase and from the zona pellucida by treatment with 0.1% trypsin. Semen collected from fertile donor (No. 11) was liquefied for 30 min at 37°C, washed three times, and adjusted in Biggers, Whitten, and Whittingham (BWW) medium supplemented with 1% bovine serum albumin (BSA, fraction V, No. A-7906, Sigma Chemical Co.) to 10×10^6 sperm/ml, and then incubated (37°C, 5% CO_2 and 95% air mixture) overnight. 18–20 h after incubation, 200 μl of capacitated sperm were collected, centrifuged (500 *g* for 5 min), resuspended in 180 μl BWW medium or 160–170 μl for neutralization experiments and incubated at 37°C for 30 min with 20 μl of the serum or hydridoma supernatant. Subsequently, 20 ova were added to this mixture and incubated under mineral oil at 37°C in an incubator as described before. After 3 h of incubation, the eggs were washed twice with BWW, fixed overnight in 10% phosphate-buffered formalin, stained with acetolacmoid and then checked for sperm penetration. Penetration was determined by the presence of swollen spermhead with a tail discernible within the cytoplasm of the ovum. Motility of sperm was recorded after overnight incubation and after incubation with ova.

For absorption experiments, the antigen preparations, namely the FA-1, affinity-purified antigens isolated using immunoinfertile serum (AAIS), and the affinity-purified antigens isolated using fertile serum (AAFS), were dialyzed overnight against BWW medium. 10–20 μl of the dialyzed antigen preparations containing 10.3–20.6 μg of the protein were incubated (37°C, 30 min) with 20 μl of the serum or hybridoma supernatant, and the resultant mixture was then incubated with 160–170 μl of sperm suspension as described above and ova were tested for sperm penetration.

Statistical analysis. Significance of differences was based on Chi-square analysis or unpaired Student's *t* test. The correlation was checked by analyzing for linear regression.

Results

The FA-1 isolated from human or murine testes used in ELISA showed a single band of 23,000 (monomer) and/or 47,000–50,000 D (dimer) in SDS-PAGE (Fig. 1, *C* and *B*, respectively). The monoclonal antibodies raised against FA-1 reacted with a specific protein band of 23,000 D on Western blot of DOC-solubilized washed human sperm (Fig. 1 *D*). The rabbit antiserum (No. A, 3) raised against purified FA-1,

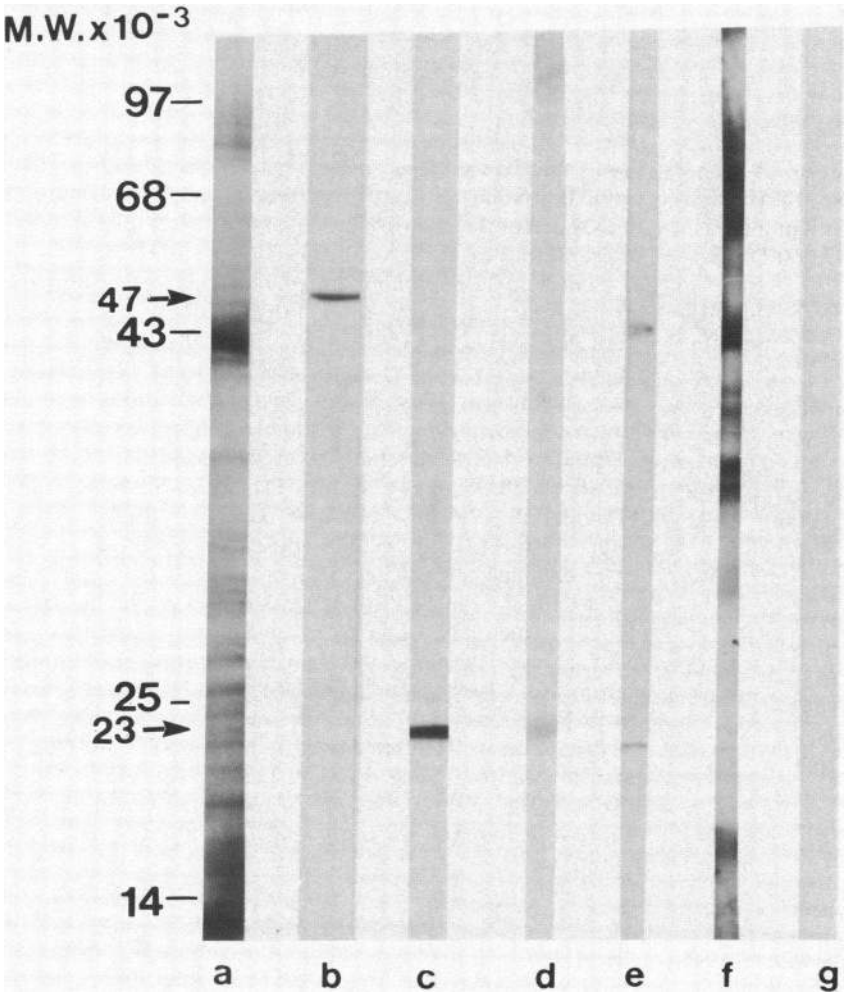

Figure 1. Specificities of purified FA-1 and its antibodies. The purified FA-1 showed a single band of 47,000–50,000 D (dimer) (*b*) or 23,000 D (*c*) in SDS-PAGE when stained with ultrasensitive silver stain. The monoclonal antibodies raised against FA-1 reacted with a single band of 23,000 D on Western blot of DOC-solubilized washed human sperm (*d*). The rabbit antiserum raised against FA-1 (No. A, 3) reacted with both monomeric and dimeric forms of FA-1 on Western blot of DOC-solubilized washed human sperm (*e*). The rabbit antiserum raised against DOC-solubilized human sperm (No. 102) recognized numerous protein bands including one in the region of 47,000 D on Western blot of DOC-solubilized washed human sperm (*f*). The control rabbit serum did not recognize any specific band on the blot (*g*). SDS-PAGE of DOC-solubilized human sperm from which blot was prepared has been included for comparison (*a*).

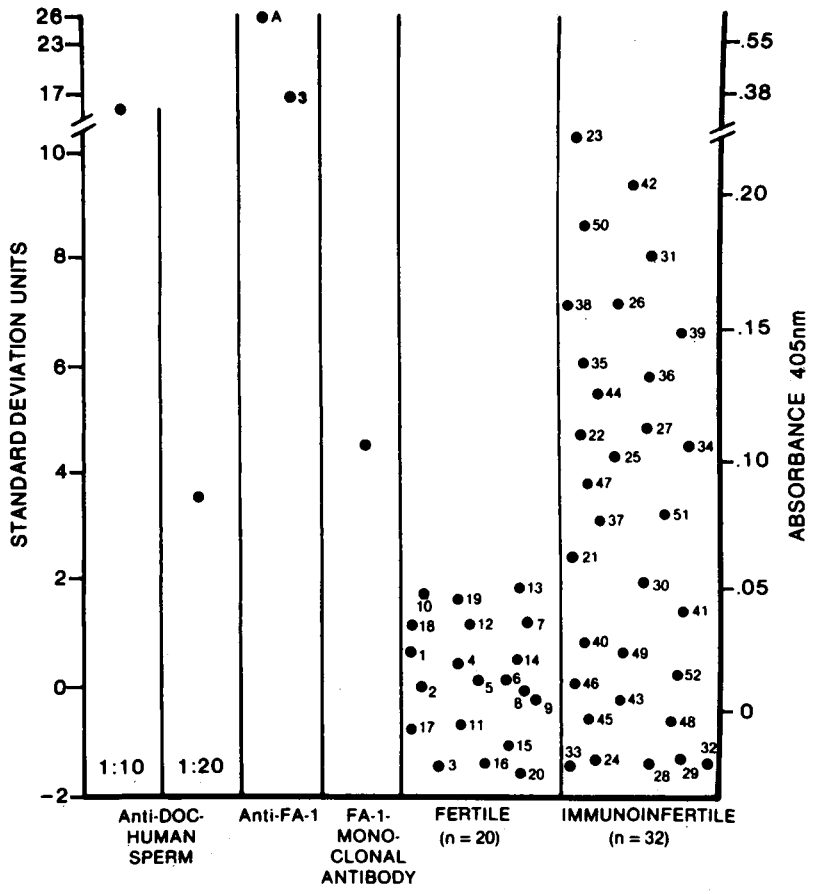

Figure 2. Reaction of the sera with purified FA-1 in the ELISA. Sera from immunoinfertile patients reacted strongly with FA-1 when compared with sera from fertile controls. Absorbance value, mean (range)±SEM; infertile sera, 0.081 (−0.051 to 0.242)±0.014; fertile sera, 0.012 (−0.040 to 0.045)±0.006; $P < 0.0001$. Rabbit anti-DOC-human sperm antiserum (No. 102) reacted strongly with FA-1, lower dilution (1:10) showing stronger reaction than higher dilution (1:20). Rabbit anti-FA-1 antiserum (No. A, 3) and monoclonal antibodies to FA-1 also demonstrated strong binding with the antigen.

reacted with both monomeric and dimeric forms of FA-1 on Western blots (Fig. 1 *E*). The rabbit antiserum (No. 102) raised against DOC-solubilized human sperm reacted with numerous protein bands, including one in the region of 47,000 D (Fig. 1 *F*).

Reaction of sera with FA-1 in ELISA. Sera from 20 fertile males and females showed either a weak or no reaction with FA-1 (absorbance value, mean [range]±SEM, 0.012 [−0.040 to 0.045]±0.006) (Fig. 2). However, when converted to SD units, none of them gave value equal to or more than two (SD units, mean [range]±SEM, 0.18 [−0.19 to 1.9]±0.282).

Table I. Comparison of Titers Obtained in Sera of Immunoinfertile Patients Through SAT, SIT, and ELISA

Serum samples	Sex	SAT titers*	SIT titers‡	Reaction in ELISA§/ mean absorbance value‖
21	F	0	1/16	.062
22	M	0	1/4	.110
23	M	1/256	1/32	.242
24	M	1/16	1/32	.018
25	F	1/16	0	.101
26	M	1/256	1/64	.162
27	M	1/1024	1/16	.113
28	M	1/64	0	−.051
29	M	1/32	0	−.019
30	M	0	1/64	.049
31	F	0	1/8	.181
32	M	0	1/32	−.028
33	M	1/256	0	−.034
34	F	1/1024	0	.105

§ Check at 1:10 dilution of serum. There was no correlation between * vs. ‖ ($\gamma^2 = 0.062$, $P > 0.39$); and ‡ vs. ‖ ($\gamma^2 = 0.057$, $P > 0.41$).

Sera from 32 immunoinfertile males and females showed a strong reaction with FA-1 (absorbance value, mean [range]±SEM, 0.081 [−0.051 to 0.242]±0.014), which differed significantly ($P < 0.0001$) from that of controls. Their SD units were significantly ($P < 0.0001$) higher than those of fertile controls (SD units mean [range]±SEM, 3.28 [−2.9 to 11]±0.67). 18 of these sera (Nos. 21, 22, 23, 25, 26, 27, 31, 34, 35, 36, 37, 38, 39, 42, 44, 47, 50, 51) reacted with FA-1 to give SD units of > 2. These sera were positive in SAT or SIT or both. However, there was no correlation observed between titers obtained through the SAT or SIT and the absorbance values observed in ELISA (Table I). For example, the serum No. 22, which had a titer of 1/4 in SIT and was negative in SAT, showed the same absorbance value (0.113) as another serum, No. 27, which had a titer of 1/16 in SIT and of 1/1,024 in SAT. 11 sera (11/32) that were negative in SAT (e.g., No. 31) and 10 sera (10/32) that were negative in SIT (e.g., Nos. 25 and 34) showed strong reactions (absorbance values, 0.181, 0.109, 0.105, respectively) with FA-1. However, fertile sera that were negative in both SAT and SIT did not react with FA-1.

Presence of anti-FA-1 antibodies and fertilization rates in IVF procedure. The presence of anti-FA-1 antibodies and the fertilization rates in the seven infertile couples have been summarized in Table II. Three of the seven infertile couples (fe-

male partners No. 31, 42, 44; and male partners No. 25, 38, 50, respectively), showed a complete inability of the husband's sperm to fertilize the spouse's oocytes in the IVF procedure (Table II). Fertilization failure was not due to agglutination or loss of motility of sperm; sperm maintained good motility and forward progression throughout the insemination period and bound to the zona pellucida of the oocytes (Fig. 3 *A*). However, the penetration into the oocytes was completely blocked and none of the oocytes (n = 14) was fertilized. Both partners of these three couples demonstrated antibodies to FA-1 (Fig. 2). In males, antibodies were present in serum and seminal plasma; in females, they were found in serum as well as in follicular fluid (Table II). Sera from these male partners (No. 38, 25, 50) reacted with protein bands of various molecular identities ranging from 14,000 to > 97,000 D on Western blot involving a DOC-solubilized human sperm (Fig. 4, *A, B,* and *C,* respectively). However, all of them reacted with a protein band in the region of 47,000–50,000 D corresponding to dimeric form of FA-1. Sera from fertile controls (No. 3, 7, 11) did not react with any protein band in this region (Fig. 4, *D, E,* and *F,* respectively). The remaining four infertile couples showed a mean fertilization rate of 75%. In these couples, the husband's sperm fertilize the spouse's oocytes (Fig. 3 *B*), which subsequently cleaved to form normal embryos (Fig. 3 *C*). In these couples, only female partners demonstrated anti–FA-1 antibodies that were present in serum as well as in follicular fluid. The male partners did not show any anti–FA-1 antibody activity in serum nor in the seminal plasma.

Analysis of sperm antigens purified using immunoaffinity chromatography. The fraction eluted at pH 2.8 from the immunoaffinity column that had immunoglobulins from serum of an infertile male (No. 38), whose sperm failed to fertilize the spouse's oocytes, demonstrated at least seven antigens of 23,000, 47,000, 50,000, 54,000, 56,000 and two of > 97,000 D, respectively, in SDS-PAGE after staining with ultrasensitive silver stain (Fig. 4 *G*). This fraction henceforth will be mentioned as AAIS. The fraction eluted at pH 2.8 from the immunoaffinity column containing immunoglobulins from serum of a fertile male (No. 11), who sired a healthy baby, demonstrated at least five antigens of 14,000, 14,800, 34,000, and 97,100 D, respectively, in SDS-PAGE after staining with silver stain (Fig. 4 *H*). This fraction, henceforth, will be mentioned as AAFS.

Effects of absorption of antibodies with FA-1 on sperm penetration assay. Both sera (tested at 10% dilution), one from an infertile male (No. 38) (SAT titer, 1/64; SIT titer, 1/8; anti–FA-1 absorbance value, 0.162) and another from an infertile female (No. 35) (SAT titer, 1/512; SIT titer 1/4, anti–FA-1 absorbance value, 0.142), inhibited human sperm penetration of zona-free hamster oocytes, when compared with fertile controls. (No. 11). The hybridoma supernatant containing monoclonal antibodies to FA-1 also inhibited SPA (penetration rates, infertile serum No. 38, 13%; infertile serum No. 35, 24%; control serum No. 11, 78%; monoclonal antibodies, 13%) (Table III). Incubation of immune sera with FA-1 before testing them in SPA, significantly ($P < 0.001$) increased the penetration rates, whereas incubation with AAFS did not show any effect on the penetration rates (Table III). Similarly, incubation of monoclonal antibodies with FA-1 or with AAIS significantly ($P < 0.001$) increased the penetration rates, while incubation with AAFS was without any effect. Incubation of control serum (No. 11) with FA-1 did not effect the penetration rates significantly. These results have been summarized in Table III.

Discussion

The results of the present investigation suggest that sera from immunoinfertile patients react strongly with the FA-1 while sera from fertile controls showed a minimal or no reaction. The minimal reactions observed with some of the fertile sera can be attributed to "immunologic background noise". There

Table II. Correlation of the Presence of Anti–FA-1 Antibodies with the Fertilization Rates in Infertile Couples

	Infertile couple												
	Female partner			Male partner									
		Anti–FA-1 antibodies*			Anti–FA-1 antibodies*		No. Oocytes Retrieved				No. oocytes fertilized	Fertilization rates	
	Patient	Serum	Follicular fluid	Patient	Serum	Seminal plasma	Total	Mat.	Int.	Imm.		Individual	Mean
												%	%
Antibodies	31	0.179	0.105	25	0.101	0.092	3	0	2	1	0	0	
in both	42	0.221	0.134	38	0.162	0.158	6	3	3	0	0	0	0
partners	44	0.130	0.101	50	0.188	0.131	5	1	4	0	0	0	
Antibodies	34	0.105	0.092	45	−0.012	−0.008	3	1	2	0	3	100	
in female	39	0.151	0.102	52	0.017	0.010	5	1	2	2	4	80	75
partner	35	0.142	0.103	53	NA	0	7	3	4	0	5	71	
only	47	0.090	0.084	54	NA	0.001	2	0	2	0	1	50	
Antibodies	$Control_1$	0	0	$Control_1$	NA	0	4	1	2	1	3	75	
in no	$Control_2$	0	−0.003	$Control_2$	NA	0	2	0	1	1	2	100	92
partner	$Control_3$	0.001	0.001	$Control_3$	NA	−0.007	2	0	1	1	2	100	

* Mean absorbance value in ELISA at 1:10 dilution. Mat, mature; Int, intermediate; and Imm, immature.

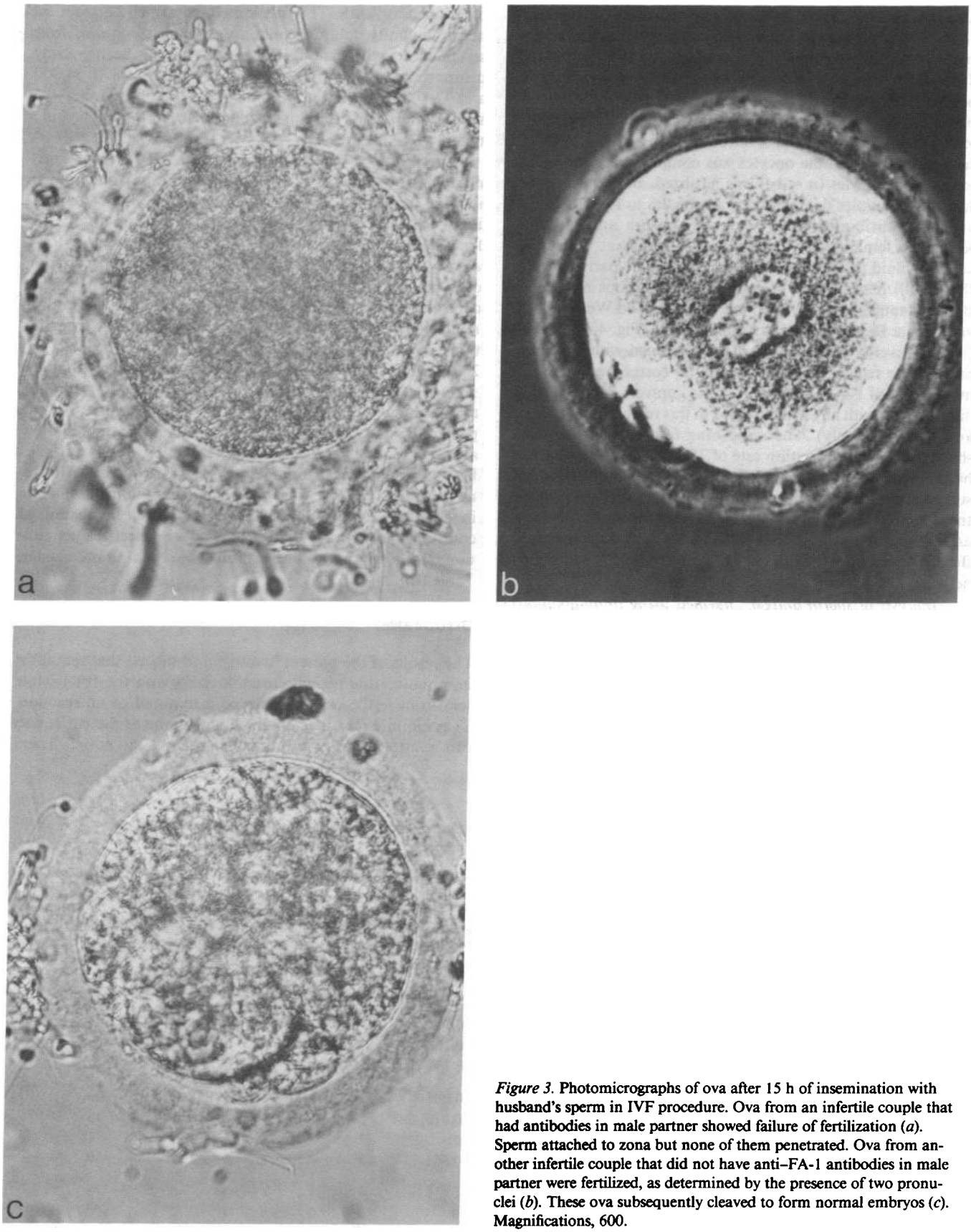

Figure 3. Photomicrographs of ova after 15 h of insemination with husband's sperm in IVF procedure. Ova from an infertile couple that had antibodies in male partner showed failure of fertilization (*a*). Sperm attached to zona but none of them penetrated. Ova from another infertile couple that did not have anti-FA-1 antibodies in male partner were fertilized, as determined by the presence of two pronuclei (*b*). These ova subsequently cleaved to form normal embryos (*c*). Magnifications, 600.

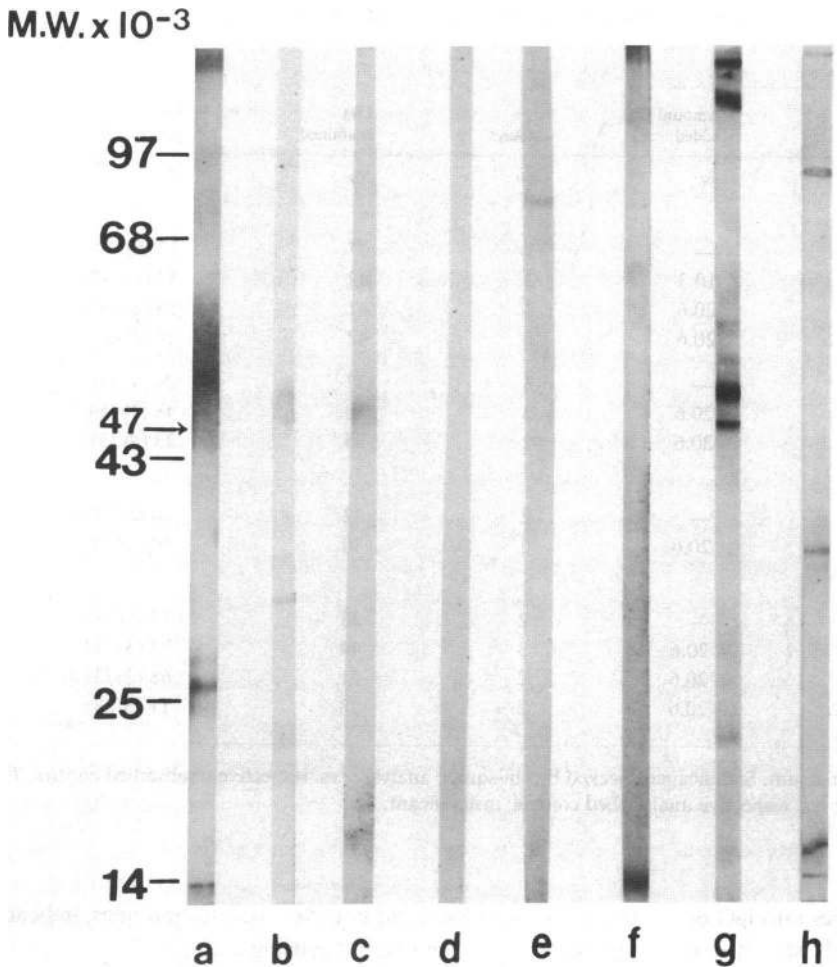

Figure 4. Reaction of sera from immunoinfertile and fertile humans with spermatozoal protein antigens on Western blot of DOC-solubilized washed sperm (*a–f*). Many of the immunoinfertile patients (e.g., No. 25, 38, 50) reacted with various protein bands including one in region of 47,000–50,000 D corresponding to the dimeric form of FA-1 (*a, b, c,* respectively). Sera from fertile controls (e.g., 3, 7, 11) did not show any specific binding in this region (*d, e, f,* respectively). The immunoaffinity-purified antigens isolated using infertile serum showed seven antigens of various molecular identities including bands in the region of 47,000–50,000 D in SDS-PAGE (*g*). These specific bands were absent in the antigens isolated using fertile sperm when checked in SDS-PAGE (*h*).

was no correlation between the titers obtained through SAT and SIT and the intensity of reaction with FA-1. This was expected because the antibodies to FA-1 interfere with the fertilization process by mechanism(s) other than agglutination and immobilization of spermatozoa. However, sera from fertile controls that were negative both in SAT and SIT showed minimal or no reaction with FA-1.

All the female partners of seven infertile couples that were positive in SAT and/or SIT, demonstrated anti–FA-1 antibodies in serum as well as in follicular fluid. But only three of these couples whose male partners also demonstrated anti–FA-1 antibodies in serum as well as in seminal plasma showed a block of fertilization process in IVF-ET procedure. We used antibody-free FCS instead of maternal serum (MS) in our IVF procedure, thus avoiding antibodies present in MS. Anti–FA-1 antibodies, monoclonal or polyclonal, are sperm-specific and do not bind to the zona pellucida or the plasma membrane of oocyte membrane (11, 12). Thus, the antibodies present in maternal follicular fluid do not bind to the oocyte and whatever quantity is carried along with the follicular fluid is diluted away during washing of the oocytes before incubating them in the insemination medium. Antibodies present in the male partner, especially in the seminal plasma, can still interfere with the fertilization process through binding on the sperm surface. The sperm-bound antibodies are not eluted by a simple washing procedure. The pH (7.4–7.8) and the osmolarity (280 mosmol/kg) of the medium is not high enough to break the antigen–antibody interactions. The anti–FA-1 antibodies allowed the sperm to bind to zonae, but penetration was completely blocked. Sperm did not display agglutination or immobilization in the insemination medium. These results are in agreement with those published by Clarke et al. (25, 26). They demonstrated that in couples where sperm antibodies were present in female partners, the replacement of MS with FCS significantly improved the fertilization rates. However, in cases where the sperm antibodies were present in male partners, replacement of MS with FCS did not improve the fertilization rates. They observed an inhibition of fertilization in IVF procedure, when 80% or more of motile sperm were antibody coated.

We did not investigate the kinetics of the immune response nor the correlation of the anti–FA-1 antibody titers with the fertilization rates. We observed a complete inhibition of fertilization in the three couples that demonstrated anti–FA-1 antibodies in female as well as male partners. The anti–FA-1 antibodies were assayed retrospectively after a block of fertilization was observed. This interesting finding needs to be confirmed in a larger population, while investigating the minimal anti–FA-1 antibody titer that can inhibit fertilization.

Our results also indicate that the anti–FA-1 antibodies when present in serum, were also detected in local genital secretions. Whether these antibodies which are present locally (follicular fluid and seminal plasma), were synthesized locally and/or extruded from the serum, cannot be concluded at the

Table III. Effects of FA-1 on Immunoabsorption of Infertile Sera in Zona-free Hamster Ova Penetration Assay

Serum		Antigen used				
Patient	Anti-FA-1 antibodies*	Source	Amount added	Assays	Ova examined	Ova penetrated
			μg	*No.*	*No.*	%
Immune sera						
38	0.162	—	—	2	47	13 (6/47)
		FA-1	10.3	2	42	33 (14/42)‡
		FA-1	20.6	3	41	59 (24/41)§
		AAFS	20.6	2	42	14 (6/42)‖
35	0.142	—	—	3	37	24 (9/37)
		FA-1	20.6	3	58	48 (28/58)§
		AAFS	20.6	2	35	23 (8/35)‖
Control sera						
11	0	—	—	2	32	78 (25/32)
		FA-1	20.6	2	30	80 (24/30)‖
Monoclonal antibodies						
MA-24	0.112	—	—	2	38	13 (5/38)
		FA-1	20.6	3	44	77 (34/44)§
		AAIS	20.6	2	31	68 (21/31)§
		AAFS	20.6	2	28	11 (3/28)‖

* Mean absorbance value in ELISA at 1:10 dilution of serum. Significance checked by chi-square analysis; ‡vs. respective unabsorbed control, $P < 0.01$; §vs. respective unabsorbed control, $P < 0.001$; ‖vs. respective unabsorbed control, insignificant.

present time. We did not classify these antibodies into IgG or IgA class. Bronson et al. (3, 27) have previously shown that these are sperm antibodies of the IgA class present on the sperm which are more potent inhibitors of fertilization in vitro.

Sera from these male partners of three infertile couples which showed a complete block of fertilization in vitro bound to various protein bands in the Western blot procedure. However, all of these sera showed binding to 47,000–50,000 D region, which corresponds to the dimeric form of FA-1. None of the sera from fertile controls bound to a protein band in this region though they bound to protein bands in other regions. These results indicate that besides the presence of antibodies to FA-1, these infertile sera also had antibodies reacting with various other sperm antigens. One (No. 38) of these infertile sera, when used in immunoaffinity chromatography, yielded at least seven antigens belonging to various molecular identities, including bands in the region of 47,000–50,000 D that were absent in the antigen fraction purified using fertile serum. These cumulative results indicate that sera of immunoinfertile patients had antibodies reacting with FA-1. However, just the presence of antibodies to FA-1 does not indicate that they are involved in infertility, especially when there are numerous antibodies present in sera which react with several other sperm antigens. We examined the role of anti–FA-1-antibodies in fertilization by immunoabsorption experiments. The immunoinfertile sera that showed reduced penetration rates in SPA demonstrated an increase after absorption with FA-1. These results can be explained by neutralization of the antibodies specifically directed against FA-1, which were otherwise causing an inhibition of the penetration rates. Incubation of control serum with FA-1 did not affect penetration rates, indicating that FA-1 per se was not causing a direct effect on sperm penetration. Interestingly, FA-1, as well as AAIS neutralized the FA-1 monoclonal antibodies that otherwise inhibited the sperm penetration. These results further confirm that immunoinfertile serum (No. 38) had antibodies to FA-1 and these antibodies were involved in causing an inhibition of the fertilization process. Similar results were obtained independently in another laboratory. In these studies, the FA-1 absorbed the antibodies present in sera of immunoinfertile patients and increased the sperm binding in a dose-dependent fashion (28).

In conclusion, our data indicate that sera from immunoinfertile patients had antibodies reacting with FA-1 and these antibodies seem to be involved in the fertilization process. Thus, the FA-1 will provide a basis for specific diagnosis and treatment of immunoinfertility in humans.

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