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Sperm Antibodies in Vasectomized Men and Their Effects on Fertilization[1]

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ABSTRACT

Sera (vbs, n = 25) and seminal plasma (vsp, n = 21) from vasectomized men (n = 25) were analyzed for cross-reaction with lithium diiodosalicylate (LIS)-solubilized human sperm extract, protamine, and fertilization antigen (FA-1) with an enzyme-linked immunosorbent assay (ELISA). Among the vbs tested, 44% reacted with human sperm extract, 28% reacted with protamine, and 44% reacted with FA-1 for at least one class of antibodies (IgG, IgA, or IgM). In contrast to the sera, the seminal plasma showed minimal reactions. Neither the vbs nor vsp were found to contain immune complexes, indicating that the antibodies were present in free form. Vasectomized sera that reacted with FA-1 showed a significant ($p<0.0001$) inhibition of human sperm penetration of zona-free hamster ova. The immunoabsorption of FA-1-positive sera with purified FA-1 significantly increased the penetration rates. Affinity-purified human immunoglobulins reactive with FA-1 and not those reactive with protamine reduced sperm penetration rates. Thus, antibodies in vbs reactive with FA-1 are relevant to infertility, causing an inhibition of fertilization. These data will have clinical relevance for diagnosis and treatment of infertility after successful vasovasostomy.

INTRODUCTION

Several studies have reported an increase in antisperm antibody formation after vasectomy in humans (Linnet, 1983; Liskin et al., 1983). Antisperm antibodies have been assayed by three methods, namely the sperm agglutination technique (SAT), the sperm immobilization technique (SIT), and the immunofluorescence technique (IFT) (Ansbacher et al., 1972; Alexander and Schmidt, 1977). These studies used whole sperm, viable or fixed, as the target antigen. There are reports indicating that postvasectomy sera react with the nuclear protamines (as detected by IFT on swollen sperm) (Tung, 1975), but not with the sperm-specific lactate dehydrogenase (LDH-C_4) (Kolk, 1979).

We recently isolated and characterized a sperm-specific glycoprotein, the fertilization antigen (FA-1), from human and murine male germ cell plasma membranes (Naz et al., 1986). The tissue-specific but species-cross-reactive monoclonal antibodies reactive with FA-1 significantly inhibit human sperm penetration of zona-free hamster ova and block penetration of murine oocytes by murine sperm (Naz et al., 1984). The FA-1 causes a reduction of fertility in actively immunized female rabbits by mechanism(s) involving an inhibition of fertilization (Naz, 1987a). The antigen is also involved in involuntary immunoinfertility in humans (Naz, 1987b, 1988).

The aim of the present study was to investigate the presence of antibodies reactive with fertilization antigen

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(FA-1) in postvasectomy human sera and seminal plasma. It was further investigated whether these antibodies were present in free form and/or as immune complexes bound to the antigen. The overall objective was to obtain information regarding the capability of antisperm antibodies in postvasectomy sera to inhibit fertilization and to search for antigens relevant to fertility.

MATERIALS AND METHODS

Antigens

Three different preparations were used to detect antibodies.

Human sperm extract (HSE). HSE was prepared by dissolving the membrane proteins of washed, ejaculated human sperm (pooled from 3 or more fertile donors) with lithium diiodosalicylate (0.3 M, LIS in 0.05 M tris(hydroxymethyl)aminomethane [Tris]-HCl, pH 8.0, containing 1 mM phenylmethylsulfonylfluoride [PMSF] and 5 mM soybean inhibitor) at room temperature for 30 min and then at 4°C for 2 h. The extract was centrifuged at 25,000 × *g* for 30 min. The LIS-solubilized sperm supernatant was dialyzed against 0.05 M Tris-HCl (pH 7.4) for 24–48 h, aliquoted and frozen at –70°C until further use.

Protamine. Salmon protamine (salmine, free-base, Grade IV; Sigma Chemical Co., St. Louis, MO) was used for immunization. Protamines are small (<10,000 MW), strongly basic proteins (pH~12) present exclusively in the nuclei of sperm. Salmon protamine immunologically cross-reacts with human protamine (Samuel, 1980). Protamine was tested for its homogeneity by acid-urea electrophoresis using 15% gel (Panyim and Chalkley, 1969). Gels were stained with Coomassie Brilliant Blue R and/or silver nitrate stain.

Fertilization antigen (FA-1). FA-1 was purified from LIS-solubilized murine testes by immunoaffinity chromatography involving monoclonal antibodies that inhibit fertilization (Naz et al., 1984, 1986). 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 MW and/or a dimer of 47,000–50,000 MW (Naz et al., 1986). Each batch of FA-1 was tested for its homogeneity. Only those batches that showed specific bands of 23,000 MW and/or 47,000–50,000 MW in sodium dodecyl sulfate (SDS)-PAGE when stained with ultrasensitive silver stain were used in the present study. In SDS-PAGE, FA-1 mainly showed its dimeric form of 47,000–50,000 MW.

Patient Sera and Seminal Plasma

Vasectomized sera (vbs, n = 25, #8–32) and vasectomized seminal plasma (vsp, n = 21, #8–32) were collected from 25 vasectomized men who had undergone vasectomy between 1979 and 1985. All men had proven fertility prior to vasectomy and none had evidence of major systemic illness or endocrine disorder. One patient was taking hydrochlorothiazide for hypertension. The average age of the group (27–45 yr) was 39 yr. The time interval from vasectomy to study date varied from 2 to 64 mo, with an average duration of 26 mo. Each man was determined to be azoospermic by means of semen analysis.

Fertile control sera (CS, n = 7, #1–7) were collected from 7 fertile men, 27–45 yr old (average: 39 yr). No control had evidence of a systemic illness or endocrine disorder and none was taking medication. All men had previously sired a healthy child.

Positive Sera

Positive sera consisted of sera (n = 5, #1001–1005) from immunoinfertile patients and rabbit antisera raised against HSE (#103), protamine (#368), and FA-1 (#A). Immunoinfertile sera were collected from infertile male patients (24–37 yr old) who demonstrated antisperm antibodies in their sera as revealed by SAT (Friberg, 1974) and/or SIT (Isojima et al., 1968). For preparing rabbit antisera, sexually mature virgin female rabbits of New Zealand white strain were injected systemically at s.c. and i.m. sites with either (a) a preparation of HSE, (b) protamine in Tris-HCl buffer (0.05 M, pH 8.0), or (c) purified FA-1 in Tris-HCl buffer (0.05 M, pH 8.0). The first injection consisted of 0.2 ml solution containing 50–75 μg of the corresponding protein emulsified with an equal volume of Freund's complete adjuvant (FCA). Two weeks after the primary injection, the rabbits were injected weekly for 3 wk with 50 μg of the antigen (protein) mixed with incomplete Freund's adjuvant (IFA). One week after the last injection, antisera were collected, aliquoted, and stored at –70°C until further use.

Enzyme-Linked Immunosorbent Assay (ELISA)

The free antibodies in serum or seminal plasma were detected using ELISA. ELISA was performed using Dynatech polyvinyl microtiter plates. Each well was incubated overnight at 4°C with 200 μl of antigen solution of either HSE (2 μg/well) or protamine (0.2 μg/well) or purified FA-1 (1 μg/well) in 0.1 M carbonate buffer (0.1 M, pH 9.6). The wells were washed 3 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 wells were incubated with PBS-Tween containing 0.25% human serum albumin (HSA) at 37°C for 45 min, and again washed 3 times with PBS-Tween. The wells were then incubated with 200μl of serum or seminal plasma (1:10 diluted in PBS-Tween containing 0.25% human serum albumin [HSA]) at 37°C for 1.5 h, washed 5 times as above, and incubated (37°C, 1.5 h) with 200 μl of affinity-purified goat anti-human immunoglobulins (Igs) (whole molecule or $F(ab)_2$) conjugated with alkaline phosphatase (α- or γ- or μ-chain specific; Cappel Lab., West Chester, PA, or Sigma Chemical Co.) diluted 1:200 (α- or γ-chain specific) or 1:700 (μ-chain specific) in PBS-Tween containing 0.25% HSA. The wells were again washed 5 times as described above and incubated with 0.2 ml of substrate solution (1 mg/l of disodium p-nitrophenyl phosphate [Sigma Chemical Co.] in 0.05 M carbonate buffer, pH 9.8). The reaction product was read at 405 nm.

Sera were run in batches of 10–15 and a known positive (#103 or #368 or #A) was included as a quality control in each batch. Each serum was assayed in duplicate and the mean value of two readings was recorded. The absorbance readings were converted to the standard deviation (SD) units with following formula: SD units = mean (test) − mean (control)/SD of control group. Test samples with SD units of ≥+2 were considered to have a positive reaction.

Detection of Immune Complexes

The immune complexes were measured using three techniques: (1) Clq binding assay, (2) Raji cell assay, and (3) polyethylene glycol precipitation method as described by Peace et al. (1980). The immune complexes were subsequently isolated and analyzed for the presence of antigen and class of antibodies.

Clq binding assay. The assay was performed by adding 0.1 ml of serum or seminal plasma to 0.1 ml of sodium ethylenediaminetetracetic acid (0.4 M, pH 7.5), incubating for 30 min at 37°C, cooling to 0°C, then mixing with 5 μl solution containing 200 μg/ml of ^{125}I-labeled Clq, and incubated again for 1 h at 4°C. The immune complexes were precipitated by adding 1.8 ml of 2.2% polyethylene glycol solution to each tube, mixing gently, and incubating for 2 h at 4°C. The immune complexes were separated by centrifugation and the radioactivities in the supernatant and the precipitin fractions were recorded.

Raji cell assay. The Raji cell technique was performed as described by Theofilopoulos et al. (1976). A sample of 100 μl of serum or seminal plasma was incubated with 1×10^6 Raji cells for 30 min at 37°C. The cells were washed (5 times) in Hank's medium and incubated with 100 μl of ^{125}I-labeled heat-aggregated human IgG (5 mg/ml) prepared from Cohn fraction-2 (Sigma Chemical Co.) to saturate the binding sites. A quantitative curve was made by adding known amounts of heat-aggregated IgG. The results were expressed as μg aggregated globulin equivalents/ml sample. Inhibition of the binding of aggregated IgG was considered evidence of the presence of immune complexes.

Polyethylene glycol precipitation. The serum or seminal plasma was mixed with polyethylene glycol to yield a final concentration of 10% polyethylene glycol. Five-tenths milliliter sample-polyethylene glycol solution was layered on a 4-ml gradient of 10% to 40% polyethylene glycol in a 5-ml cellulose nitrate tube. The resultant solution was centrifuged in an ultracentrifuge in a SW 50 rotor at 10,000 rpm for 20 min–1 h, and the gradient solution was separated into 0.25-ml fractions using the fraction recovery system. These fractions were examined with a long wavelength 365 mercury line lamp for the precipitant. Positive fractions were washed briefly with approximately 0.5 ml phosphate-buffered saline (PBS) and spun on a table-top centrifuge for 10 min at low speed and the precipitates were collected.

Each sample was tested at least twice on different days. Negative fertile control sera as well as positive control sera from patients with systemic lupus erythematosus were included in each assay. Sera from vasectomized rabbits (n = 3) and from age-matched male rabbits of proven fertility (n = 3) were also analyzed. Sera from previously fertile rabbits were collected 85–90 days postvasectomy.

Analysis of antigens and antibodies present in the immune complexes. The immune complexes, isolated as

above, were dissociated by dissolving them in citrate buffer (0.33 M, pH 2.8) (Phillips and Draper, 1975). The separated components were electrophoresed into the agarose gel, which contained 5 times the normal suggested buffer used in immunoelectrophoresis (0.5 M sodium acetate barbital, pH 8.6) to neutralize the citrate. After 1 h of electrophoresis, the gel was removed and 2 wells were made. One well was made on the anode side and the second on the cathode side. The antigen (protamine or FA-1) was placed on the cathodal well and the antibody (goat anti-human or goat anti-rabbit IgGs (γ-chain or μ-chain specific) was placed in the anodal well. Diffusion was allowed to occur overnight. Positive reactivity was determined by the presence of a precipitin band.

Sperm Penetration Assay (SPA)

Zona-free hamster ova-human sperm penetration assay (SPA) was performed as described by Yanagimachi et al. (1976) and modified by Syms et al. (1985). Superovulation was induced in adult female golden hamsters by injecting them i.p. with 30 IU pregnant mare's serum gonadotropin (PMSG, Sigma Chemical Co.) on Day 1 of the cycle. After 55–72 h, 25 IU human chorionic gonadotropin (hCG, Sigma Chemical Co.) was administered i.p. The animals were killed 15–17 h after hCG injection and mature unfertilized ova were collected from the oviducts. The ova were separated from the surrounding cumulus cells by incubation with 0.2% hyaluronidase in Biggers, Whitten and Whittingham medium (BWW) and from the zonae pellucidae by treatment with 0.1% pancreatic trypsin in BWW. The zona-free ova were washed twice in BWW and placed in the center of tissue culture dishes.

Semen from fertile men was allowed to liquefy for 15–30 min at 37°C, and then was combined with an equal volume of prewarmed (37°C) test yolk buffer to a homogeneous mixture. The mixture was placed in a refrigerator (4°C) for 42–48 h to allow the sperm to capacitate. The mixture was centrifuged, supernatant was discarded, and the sperm pellet was subjected to thermal shock by immersing the tube in water at 37°C. The sperm pellet was washed twice with 6–10 ml BWW supplemented with 1% bovine serum albumin (BSA, fraction V, No. A-7906, Sigma Chemical Co.), then gently resuspended and overlayed with 0.2–0.6 ml 1% BSA containing BWW. The sperm concentration was adjusted to 5×10^6 sperm/ml, and a 50-μl drop of the sperm suspension was placed in the center of a tissue culture dish (Falcon 3001, 35 × 10 mm, Falcon Plastics, Los Angeles, CA) containing zona-free ova covered with mineral oil. The sperm and ova were incubated together for 2.5 h at 37°C in air under oil; ova were washed twice in BWW and stained immediately. For staining ova were kept for 2 min in the acridine orange (0.2%) stain-diluted (1:1) in 1% BSA-BWW. After staining, ova were washed in 1% BSA-BWW, transferred onto a microslide, and covered with a coverslip using vaseline-paraffin (25:1) mixture. Penetration was determined by the presence of swollen spermhead in the cytoplasm of the ovum. The number of sperm bound to and penetrating the ova were recorded. Motility of sperm was recorded before and after incubation with ova.

For investigating the effects of sera (heat-inactivated, (56°C for 30 min) on SPA, they (10%) were incubated at 37°C for 45 min with capacitated sperm (5×10^6 sperm/l); the sperm were then added to the zona-free ova as described above. The Igs (purified as discussed below), were tested at the final concentration of 40–60 μg protein per 100-μl drop containing sperm and ova. The assay was repeated at least 2–3 times using three different fertile donors; each sample was tested with at least 20–50 ova.

For immunoabsorption experiments, the antigen preparations–namely the FA-1 or BSA–were dialyzed against 0.05 M PBS (pH 7.4), and lyophilized. The sera (#14 and #16) that reacted with FA-1 were incubated with lyophilized FA-1 or BSA for 30 min at 37°C and incubated overnight at 4°C. The unabsorbed or immunoabsorbed sera were tested at 10% final dilution as described above. The immunoabsorption was performed in such a way that 5 μl of serum was absorbed with 12.1 μg or 16.5 μg protein (FA-1 or BSA).

Immunoaffinity Purification of Antibodies

Immunoaffinity chromatography was performed as described elsewhere (Naz et al., 1986). The vasectomized sera (#8 and #16) were precipitated with 18% Na_2SO_4, and the IgG fraction was separated by *O*-diethylaminoethyl (DEAE)-cellulose chromatography. The antigen (FA-1 or protamine) was coupled to Sepharose-4B activated by *CN*Br, and the coupled gels were packed into a column and equilibrated with 0.1 M phosphate buffer (pH 8.0). The Igs (1 ml containing

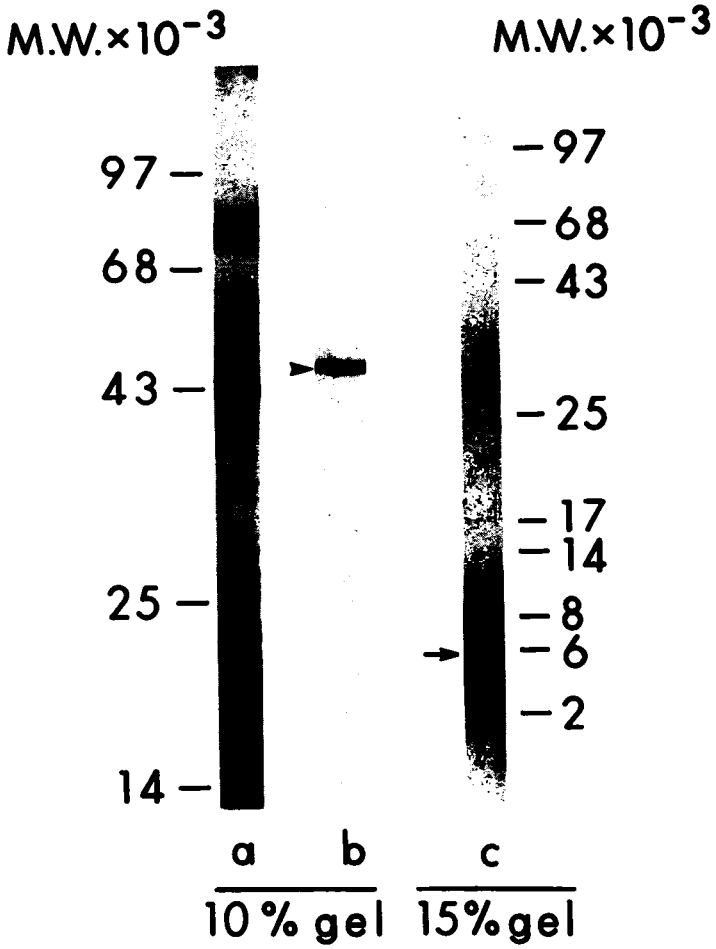

FIG. 1. SDS-PAGE pattern of the antigenic preparations stained with silver nitrate. FA-1 showed a single band *(arrowhead)* of 47,000–50,000 MW (dimer) *(Lane b)*. Human sperm extract demonstrated numerous bands *(Lane a)*. Protamine, when run in 15% acid-urea gel, demonstrated a thick band containing two polypeptide bands *(arrow)* between 6000 and 8000 MW *(Lane c)*.

2–3 mg of protein) separated from vbs #8 were incubated with the protamine-Sepharose 4B column, and the Igs separated from vbs #16 were incubated with FA-1-Sepharose-4B column for 3 h at room temperature. The unbound Igs were washed at pH 8 (0.1 M phosphate buffer) and the 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 for 48 h against PBS, and frozen (–70°C) until investigated in SPA.

Western Blot Procedure

Antigenic specificities of the antisera were evaluated by the Western blot procedure. Briefly, a 2.5- to 3.5-mg protein solution of human sperm extract was run under nonreducing conditions in the slab SDS-PAGE (10% gel) (Laemmli, 1970), transferred to nitrocellulose paper (Towbin et al., 1979), and reacted with sera (1:50). The reacted antigens were localized by the procedure described elsewhere (Naz et al., 1983).

Statistical Analysis

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

RESULTS

The immunoaffinity-purified FA-1 showed a single band of 47,000–50,000 MW (dimeric form) in SDS-PAGE (Fig. 1, Lane b), and HSE demonstrated numerous bands of various molecular identities (Fig. 1, Lane a). Protamine showed a broad band consisting of two peptides between 6000 and 8000 MW in acid urea gel electrophoresis (Fig. 1, Lane c).

The vbs (n = 25) and vsp (n = 21) collected from 25 vasectomized men were investigated for their reactivity with HSE, protamine, and FA-1. Among the 11 sera reactive with HSE, 4 were positive for IgG, 8 for IgA, and 3 for IgM; of these, 3 (#8, #18, and #27) were positive for both IgG and IgA and 1 (#27) was positive for both IgA and IgM classes (Fig. 2a,b, and c). Among the 7 sera reactive with protamine, 2 were positive for IgG, 6 for IgA, and 3 for IgM; of these, 1 (#11) was positive for both IgG and IgA, and 1 (#8) showed positive reaction for all three classes. Among the 11 sera reactive with FA-1, 7 were positive for IgG, 1 for IgA, and 7 for IgM antibodies; of these, 3 (#20, #24, #25) were positive for both IgG and IgM, and 1 (#27) was positive for both IgA and IgM. None of the sera was positive for all three classes of antibodies. One vbs (#27) contained IgA antibodies, and another (#14) had IgM antibodies reacting with all three antigenic preparations (Fig. 2a, b, and c). Sera from infertile men had a significantly higher IgG antibody response and a lower IgA antibody response for all three antigenic preparations, compared to sera from vasectomized men (Fig. 2a and b). Antibodies reactive with HSE or protamine mainly belonged to the IgA class, whereas antibodies reactive with FA-1 belonged mainly to IgG or IgM class.

In contrast to vbs, vsp showed little reaction with any antigen. Among the 5 vsp reactive with HSE, 5 were positive for IgG, 2 for IgA, and none for IgM; of these, 2 (#12, #27) were positive for both IgG and IgA.

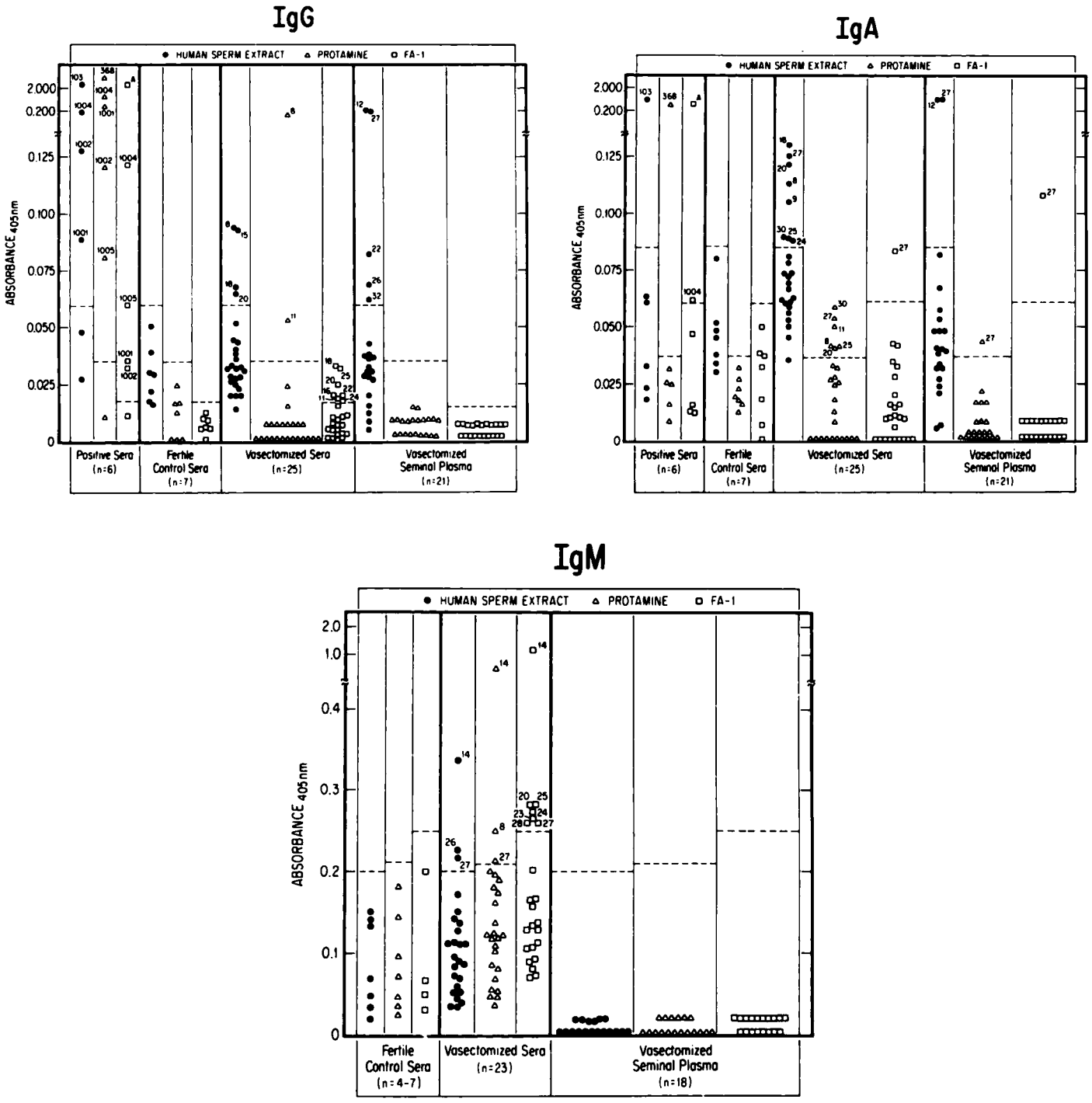

FIG. 2. *a)* Reaction of the IgG antibodies in vasectomized sera and seminal plasma in ELISA. Sera from vasectomized patients reacted strongly with these antigenic preparations compared to sera from fertile controls. The values above the line (- - - -) are >2 SD units compared to the corresponding values of fertile control group. None of the seminal plasmas from vasectomized men reacted with protamine or FA-1. Sera from immunoinfertile men reacted strongly with all the three preparations compared to the sera from vasectomized men. Rabbit anti-human sperm extract (HSE) antiserum (#103), rabbit anti-protamine antiserum (#368), and rabbit anti-FA-1 antiserum (A) reacted very strongly with the corresponding antigen.

b) Reaction of the IgA antibodies in vasectomized sera and seminal plasma with HSE, protamine, and FA-1 in ELISA. Sera from vasectomized men reacted strongly compared with the sera from fertile men. The values above the line (- - -) are >2 SD units compared to the corresponding values of the control group.

c) Reaction of IgM antibodies in vasectomized sera and seminal plasma with HSE, protamine, or FA-1 in ELISA. Though some of the sera reacted with all the three antigenic preparations, none of the seminal plasma reacted with any of the antigenic preparation. The values above the line (- - -) are >2 SD units compared to the corresponding values of the control group.

TABLE 1. Analysis of circulating immune complexes.

Sample	(n)	Immune complexes			Analysis of immune complexes	
		Clq[a]	Raji cell[b]	PEG (band)[c]	Antigen(s)	Antibody
Vasectomized human sera[d]	9	< 4	< 50	0	–	–
Vasectomized human seminal plasma[e]	7	< 4	< 50	0	–	–
Fertile human sera	5	< 4	< 50	0	–	–
Systemic lupus erythematosus patient sera	5	38–46	175–230	1–3	DNA and histone	IgG
Vasectomized rabbit sera[f]	3	1–19	–	0–1	FA-1 and protamine	IgG
Fertile rabbit sera	3	3– 6	–	0	–	–

[a]Expressed as % inhibition, normal range 0–6%.

[b]Expressed as μg/ml binding, normal range 0–65 μg/ml.

[c]PEG = polyethylene glycol precipitation.

[d]Five of these vasectomized sera showed reaction (>2 SD units) with at least one of the antigenic preparation, and the remaining four did not react with any antigen.

[e]Two of these vasectomized seminal plasma showed reaction with (>2 SD units) at least one of the antigenic preparation, and the remaining five did not react with any antigen.

[f]Of three rabbits sera tested, one did not show any band (with PEG) and other two showed one band each, both reacting with FA-1 and protamine.

None of the vsp except one (#27) correlated with the presence of antibody in the vbs; this vsp reacted with protamine and FA-1 for IgA antibodies and showed IgG antibodies reactive with all three antigenic preparations. None of the vsp had IgM antibodies.

The vbs (n = 9) and vsp (n = 7) were analyzed for the presence of immune complexes. None of the sera with (#8, #11, #18, #20, #25) or without (#10, #13, #19, #30) ELISA-detectable free antibodies were found to contain immune complexes (Table 1). Similar results were obtained with vasectomized seminal plasma. None of the vsp with (#12, #27) or without (#8, #10, #11, #13, #19) ELISA-detectable free antibodies were found to have immune complexes. In contrast, 2 of the 3 sera from vasectomized rabbits (n = 3) were found to have immune complexes. Analysis of the immune complexes revealed that both sera contained FA-1 and protamine and were found to have Igs of the IgG class (Table 1). On the contrary, immune complexes were absent in the sera of nonvasectomized fertile rabbits. The vbs (n = 6) were investigated for their effects on human sperm penetration of zona-free hamster ova (Table 2). A significant ($p<0.0001$) reduction in the number of sperm penetrated per oocyte and in the percentage of ova penetrated was seen in 3 sera reactive with FA-1 (and not reactive with protamine or HSE), as compared to fertile control sera (Fig. 3 and Table 2). Two of these sera (#16 and #22) were positive (>2 SD units) for IgG antibodies and one (#14) for IgM antibodies. All of these sera were positive for the sperm immobilization and sperm agglutination antibodies.

Both sera (#14 and #16) that reacted with FA-1 and reduced penetration rates showed a significant ($p<0.001$) increase in penetration rates after incubation with FA-1 (Table 2). Incubation of these sera with an equivalent quantity of BSA did not show any effect on the penetration rates (data not shown).

The human IgG antibodies isolated from vbs #16 using FA-1-affinity column inhibited SPA. In contrast, the human Igs isolated from vbs #8 using protamine-affinity column did not affect SPA (Table 2). Rabbit antiserum raised against protamine (#368) (Fig. 2a) also did not inhibit SPA (Table 2). In contrast, rabbit antiserum raised against FA-1 (#A) (Fig. 2a) showed a significant ($p<0.0001$) reduction in SPA, the effects being more drastic when purified IGs were used (Table 2). Three vbs (#8, #15, and #30) not reactive (<2 SD units) with FA-1 but reactive (>2 SD units) with HSE also showed a reduction in the number of sperm penetrated per egg and/or percentage of ova penetrated (Table 2). However, the effects were not as strong as those observed with sera reactive with FA-1.

In the Western blot procedure involving HSE, the vbs that inhibited SPA (#16 and #28) and were reactive only with FA-1 in ELISA showed binding in the region corresponding to the dimeric form of FA-1 (47,000–50,000 MW) and in the regions of 16,000 MW and >97,000 MW (Fig. 4, lanes g and h, respectively). The vbs (#8, #15, and #30) that showed some degree of inhibition in SPA and did not react (<2 SD units) with

TABLE 2. Effects of sera on human sperm penetration of zona-free hamster ova.

Serum (#)	Antibody titer		FA-1 used (μg)	Sperm penetrated per egg (Mean ± SD)	Ova penetrated (%)
	Immob.	Agglut.			
Vasectomized sera					
a. Sera reactive with FA-1[a]					
14	1:8	0	–	0.38 ± 0.73*	27*
14	1:8	0	12.1	2.80 ± 3.10†	38†
14	1:8	0	16.5	3.90 ± 2.80	49†
16	0	1:64	–	0.58 ± 0.90*	37*
16	0	1:64	12.1	3.80 ± 4.10†	49†
16	0	1:64	16.5	6.20 ± 1.90†	61†
22	1:16	1:128	–	0.95 ± 1.48*	47*
b. Sera not reactive with FA-1 but reactive with LIS-sperm[b]					
8	0	1:64	–	5.20 ± 2.10*	100
15	0	0	–	6.30 ± 4.00*	100
30	1:8	1:32	–	2.00 ± 1.90*	68*
Affinity-purified human antibodies					
a. Anti-FA-1	0	0	–	2.40 ± 1.90*	61*
b. Antiprotamine	0	±	–	10.80 ± 4.90	100
Fertile sera	0	0	–	18.40 ± 10.60	100
Rabbit antisera					
a. Antiprotamine	0	0	–	20.00 ± 6.00	100
b. Anti-FA-1					
1. Serum	0	0	–	2.10 ± 2.10*	69*
2. Purified immunoglobulins	0	0	–	0.37 ± 0.12*	25*

[a]Sera that showed ≥ 3 SD units for at least one class of antibody (IgG or IgA or IgM) in ELISA.

[b]Sera that showed < 2 SD units with FA-1 and > 2 SD units with LIS-sperm for at least one class of antibody (IgG or IgA or IgM) in ELISA.

*Vs. fertile controls, $p<0.0001$, others were insignificant vs. fertile controls.

†Vs. unabsorbed controls, $p<0.0001$.

FA-1 but reacted (>2 SD units) with HSE in ELISA showed binding with various proteins other than in the region of 47,000–50,000 MW (Fig. 4, Lanes d, e, and f, respectively). Interestingly, all of these vbs showed binding in the region of 18,000–20,000 MW. The rabbit antiserum raised against HSE (#103) showed binding with at least 11 protein bands, including in the regions of 47,000–50,000 MW and 18,000–20,000 MW (Fig. 4, Lane a). The rabbit antiserum raised against FA-1 (#A) recognized a prominent band of 47,000–50,000 MW (Fig. 4, Lane b). The fertile control serum (#7) reacted with three bands of 27,000, 82,000, and 97,000 MW (Fig. 4, Lane c).

DISCUSSION

The vbs had antisperm antibodies that react with the sperm surface glycoprotein FA-1 as well as the nuclear protein protamine. These antibodies belong to all three classes of Igs, namely IgG, IgA, and IgM. Overall, 44% (11/25) of the vbs were positive for HSE, 28% (7/25) for protamine, and 44% (11/25) for FA-1 in at least one class of antibodies. The immune response observed with postvasectomy sera was weaker than that observed with infertile sera. The immunoinfertile male sera showed a greater IgG response with all the three antigenic preparations, whereas vasectomized male sera showed a higher IgA response.

In contrast to sera, there were only a few vsp that were positive for HSE (20%) or protamine (4%) or FA-1 in IgG or IgA antibody tests. None of the vsp contained sperm-reactive antibodies belonging to the IgM class. These data agree with those published earlier by Ansbacher (1973), who found that all 39 ejaculates investigated at one year postvasectomy were negative for agglutinins, tested against whole sperm.

There was no correlation between the presence of antibodies in vbs and in vsp. The vasectomized patients with antibodies in seminal plasma did not necessarily have antibodies in serum and vice versa. The seminal plasma collected for vasectomized men did not include secretions from the proximal part of the genital tract. These results indicate that a majority of the serum antibodies probably enter seminal plasma via rete testis or epididymis, rather than through the vesicular glands or prostate. Also, if there is a localized antibody production, it must take place in the proximal genital tract. Alternatively, antibodies may have combined with the

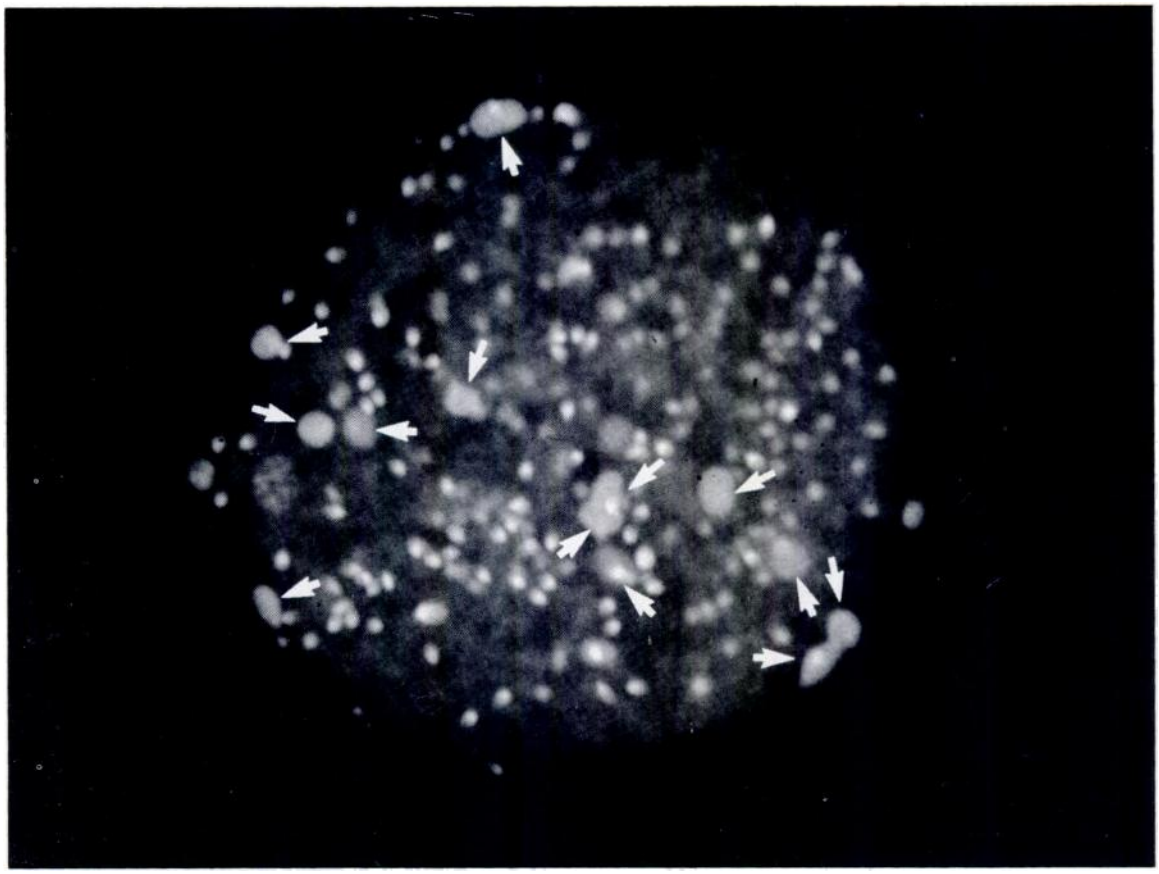

FIG. 3. Photomicrograph of a zona-free hamster oocyte penetrated with human sperm and stained with acridine orange. In this oocyte, >100 sperm were attached and 13 sperm penetrated as demonstrated by swollen spermheads *(arrows)*. These sperm were exposed to vasectomized blood sera #15, which was positive for antibodies against human sperm extract but was negative for antibodies against FA-1.

superphysiologic dose of sperm antigens developed after vasectomy to form immune complexes, rendering them undetectable by ELISA. However, analysis of the vbs and vsp with or without ELISA-detectable free antibodies indicated no immune complexes after repeated testing by several methods. In contrast to human vbs, the sera (67%) from vasectomized rabbits demonstrated immune complexes that contained FA-1 as well as protamine. These data suggest that the rabbit is more susceptible to the formation of immune complexes after vasectomy. Bigazzi (1981) found a strong antisperm antibody response with the formation of immune complexes after vasectomy in rabbits. Thus, whatever immune response to sperm was elicited after vasectomy of humans was not present as immune complexes.

The sperm surface glycoprotein, FA-1, might be important in infertility. Sera that were positive for FA-1 showed a significant reduction in penetration rates in SPA. The penetration inhibition activity was present in both IgG and IgM classes (the classes tested). That antibodies to FA-1 can cause reduction of penetration rates is evident from the affinity-purification, immunoabsorption experiments, and active immunization studies. The anti-FA-1 human Igs purified from a vbs using FA-1-immunoaffinity column, and the rabbit anti-FA-1 Igs reduced penetration rates. The sera that reduced penetration rates showed a significant increase after absorption with FA-1. We have shown that active immunization of female rabbits with species-cross-reactive FA-1 causes a significant reduction in fertility (Naz, 1987a). The murine antibodies (monoclonal and polyclonal) reduce penetration rates in SPA and block murine *in vitro* fertilization (Naz et al., 1984).

The results of affinity purification and active immunization studies indicate that the antiprotamine antibodies are not relevant to infertility. The rabbit an-

FIG. 4. Reactions of sera with spermatozoal antigens on Western blot of human sperm extract (HSE). The vasectomized sera reacted with numerous protein bands corresponding to various molecular identities (Lanes d–h). The sera (#16 and #28) that showed a strong reaction (SD units≥ 2) with FA-1 in ELISA demonstrated reaction with a protein band in the region 47,000–50,000 MW corresponding to dimeric form of FA-1 *(Lanes g* and *h)*. Three vasectomized sera (#8, #15, #30) that did not show a positive reaction with FA-1 (but showed a positive reaction with HSE) demonstrated binding with a low molecular weight protein band in the region 18,000–20,000 MW *(Lanes d–f)*. Fertile control serum (#7) did not react in regions 47,000–50,000 MW and 18,000–20,000 MW *(Lane c)*. Rabbit anti-FA-1 antiserum (#A) reacted with a single band corresponding to the dimeric form of FA-1 *(arrowhead) (Lane b)*, whereas rabbit anti-HSE antiserum demonstrated binding with numerous antigens, including in the regions corresponding to FA-1 and the low molecular weight protein (18,000–20,000 MW) *(Lane a)*. The gels were calibrated using a mixture of protein standards of known molecular weights.

tiprotamine antibodies did not affect penetration rates in SPA. Similarly, the antiprotamine human Igs purified from vbs by protamine-affinity column were not effective in reducing penetration rates. These antibodies did not react with human sperm plasma membrane although they reacted strongly with human swollen spermheads (data not shown). Active immunization of female rabbits (n = 23) with various doses of protamine also did not affect fertility, though the animals developed very high titers of antiprotamine antibodies in serum as well as locally in genital tract secretions (Naz, unpublished data). Since protamine is a nuclear protein, antibodies to it will not attach to the sperm surface and follow en route to fertilization. However, sensitization against protamine can cause antifertility effects through induction of cell-mediated immune factors (lymphokines and monokines) resulting in spermicidal and embryotoxic effects (Naz and Mehta, 1989).

There may be autoantigens on the sperm surface other than FA-1 that are relevant to fertility. Sera containing antibodies reactive with sperm surface components other than FA-1 showed some effects on penetration rates, although minimal. As evidenced by the Western blot procedure, these sera did not show any reaction with FA-1. However, they reacted with various antigens, and all of them showed binding in the region of 18,000–20,000 MW. The significance of reaction of the fertile control serum with 3 bands of 27,000, 82,000 and 97,000 MW is not known. These may be nonspecific immune reactions observed with few sera. However, this indicates that these antigens probably are not relevant to fertility.

In conclusion, our data indicate that autoantibodies to sperm cell components generated after vasectomy belong to all three classes of immunoglobulins (IgG, IgA, and IgM) and are mainly present in free form rather than as immune complexes. The autoantibody response to FA-1 is relevant to infertility resulting in inhibition of fertilization. This is the first study indicating involvement of an antigen postvasectomy that is relevant to human infertility. These data will have clinical applications in diagnosis and treatment of infertility observed in 30%–60% of cases after successful vasovasostomy (Jarow et al., 1985).

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