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I. IDENTIFICATION OF ANTIGENS

Sera from couples suffering from a minimum of two years of primary infertility were tested for their capacity to impede normal human sperm function by agglutinating or immobilizing them. Those sera found positive for antibodies that could strongly agglutinate or immobilize normal human sperm were selected for further screening. Those sera that could not agglutinate or immobilize normal human sperm were discarded.

In order to ascertain that these subjects were free of any autoimmune disorders, the sera of subjects were screened for antibodies to normal human somatic tissues. The relevant couple were also examined physically for the presence of any anatomical disorders relevant to infertility. These included:

a. History of any physical trauma to the reproductive system by any form of infection in the reproductive tract, orchitis, herniation, testicular tumor or vasectomy in the male.

b. Block of the uterus or Fallopian tubes, or ovarian cysts as revealed by hysterosalpingography.

Those subjects found negative for both autoimmune disorders and anatomical disorders were selected for further analysis. Sera containing autoantibodies to normal human tissue were discarded. Sera from subjects having any history of anatomical disorders relevant to infertility were also eliminated.
Antibodies in sera positive for agglutinating or immobilizing normal human sperm were investigated for their pattern of localization on sperm by indirect immunofluorescence. Those sera that were found negative in indirect immunofluorescence analysis against normal human sperm were discarded. Sera positive for indirect immunofluorescence studies on sperm were used to identify antigens on human sperm extracts by western blotting. These antigens recognized on the human sperm in western blots and the antibodies in infertile sera recognizing these antigens were further characterized. Sera found negative for any one of the above tests with the exception of autoimmune disorder screening were discarded.

A brief picture of methodology and criteria used for identification of antigens using sera from the infertile couple is depicted in the flow chart on the following page. Based on this screening pattern, sera from one couple (out of 23) selected for further analysis. The sera from the infertile couple selected for study satisfied above criteria. Following is a detailed picture of materials and methods used.

A. Infertile Subjects

Reports of clinical history of the male and female partner were recorded. This included number of years of infertility and brief medical history with primary
INFERTILE SERA
(TWO YEARS PRIMARY INFERTILITY)

**STEP I**
AGGLUTINATING OR IMMobilIZING CAPACITY OF NORMAL HUMAN SPERM BY SERA

DISCARD SERA ← - ve
+ ve (MORE THAN TWO BUNCHES AT X200 MAGNIFICATION OR MORE THAN 80 % IMMOBILE)

**STEP II**
SCREEN COUPLE FOR
* ANATOMICAL DISORDERS
* AUTOIMMUNE DISORDERS

DISCARD SERA ← + ve
- ve

**STEP III**
INDIRECT IMMUNOFLUORESCENCE ASSAY ON NORMAL HUMAN SPERM
* IN SUSPENSION
* FIXED IN METHANOL

DISCARD SERA ← - ve
+ ve

[HEAD]

[**PATTERN**] → MIDPIECE

[**MAIN TAIL PIECE**]

**STEP IV**
WESTERN BLOT OF NORMAL HUMAN SPERM EXTRACTS WITH SERA

IDENTIFICATION OF PROTEIN BANDS FOR FURTHER CHARACTERIZATION
relevance to infertility. Physical trauma to the reproductive system by any form of infection in the reproductive tract, orchitis, herniation, testicular tumor or vasectomy in the male subject was taken into consideration. Block of the uterus or Fallopian tubes, or ovarian cysts as revealed by hysterosalpingogram and laparoscopy in the female partner were also recorded.

1. Semen analysis

The initial evaluation of the male subject included a semen analysis. For semen analysis at least three specimens were obtained. A period of five days abstinence was recommended prior to collection of sample (Mortimer et al. 1982). Sample was collected by masturbation without the use of any lubricants or condoms in a 50 ml graduated plastic tube (Falcon 2070 Blue max, Becton Dickinson, Lincoln park, NJ). Semen sample was allowed to liquefy at room temperature for 30 min. after which parameters including volume, sperm count, sperm motility and membrane anomalies were assessed according to the WHO protocols (WHO 1980 Laboratory manual for the examination of human semen and semen-cervical mucus interaction: WHO special program of research, development and research training in human reproduction press Concern, Singapore).

a. Semen volume: Semen volume was measured after liquefaction directly in the graduated tube in which the sample was collected.
b. **Sperm motility**: Motility was determined both quantitatively and qualitatively. The estimation was done on a wet preparation of semen. A drop of semen was placed on a clean dry slide and covered with 22 mm coverslip for optimal viewing at a higher magnification.

**Quantitative determination of motility**: Using a magnification of X200, at least 10 randomly selected microscopic fields were examined under a Nikon Optiphot microscope. At least 100 sperm were counted and the result expressed as % motility (number motile against number immotile within a count of hundred). The analysis provided the number of motile sperm regardless of the quality of movement.

**Qualitative determination of motility**: The subjective assessment of motility was done by grading the forward progression carried out by majority of sperm.

- **None** = most sperm showed no forward progression
- **Poor** = most sperm showed weak forward progression
- **Good** = most sperm showed moderate forward progression
- **Excellent** = most sperm showed active forward progression

Both quantitative and qualitative assessments were done at 1h and 3h intervals after collection of specimen samples.

c. **Sperm count**: Sperm count was estimated on a wet preparation in a Neubauer's chamber. Semen was diluted 1:100 in double distilled water (DDW) containing a drop of 37 % (w/v) formalin for fixing the sperm to facilitate
counting. A drop of diluted sample was transferred to the haemocytometer and covered with a coverslip. Sperm were counted under a phase contrast microscope at X200 magnification in the White Blood Corpuscles (WBC) chamber consisting of 16 squares. Sperm concentration was calculated by the following formula:

\[
\text{Sperm conc.} = \frac{\text{No. of sperm counted in all 16 squares}}{16} \times \text{multiplication factor (10,000)} \times \text{dilution factor (100)} \text{ per ml}
\]

d. **Hyposmotic swelling test (HOST)**: HOST was performed according to the procedure of Jeyendran et al. (1984). HOST aids in determining the fertilizability of sperm in a semen sample. For this test, semen was allowed to liquefy and highly motile sperm was obtained from this preparation by a swimup procedure (Described elsewhere in this part of Materials & Methods). 100 µl sperm was incubated with 100 µl of HOS solution (0.15M fructose and 0.05M sodium citrate in a ratio of 1:1) for 60 min. at 37°C.

A drop of the above sample was placed in a glass slide and observed under phase contrast at a magnification of X400 for curling of sperm tails (Sperm swelling) and counted. A minimum of 100-200 sperm was counted per sample and the percent swelling calculated.

\[
\% \text{Swelling} = \left( \frac{\text{Swollen sperm}}{\text{Total sperm counted}} \right) \times 100
\]

A sperm that swells in HOST as shown by a curling tail is normal and indicates that integrity of membrane...
is intact. In a normal semen sample greater than 60% of sperm should swell up. Lesser than 50% swelling is an indication of abnormal membrane integrity.

2. Serum analysis

Samples of blood from both the male and the female subjects were obtained by venipuncture and serum separated and frozen at -70°C until use. The serum from the male was designated as IF-10 and serum from the female as IF-11.

a. Screening for antibodies against non-germ cell antigens: Both IF-10 and IF-11 were first analyzed for antibodies to somatic tissues, nuclear proteins, DNA, mitochondria and parietal cells by indirect immunofluorescence on frozen human tissue sections and for antibodies to thyroglobulin and cardiolipin by latex agglutination.

b. RIA for hormones: Serum levels of testosterone, follicle stimulating hormone (FSH) and luteinizing hormone (LH) were measured by radioimmunoassay against WHO standards according to WHO reagent program protocols.

**FSH and LH**: RIA for LH and FSH was performed using the double antibody technique. The assay was set up in triplicate in 10 x 75 mm glass tubes. The following constituents were added in the order:

- 400 μl assay buffer (PBS 50mM, pH 7.4 with 0.5% BSA, 0.025 M EDTA and 0.1% sodium azide),
- 100 µl standard hormone or unknown samples,
- 100 µl labeled hormone and
- 100 µl antiserum (anti-FSH or LH).

Whenever any component of assay system was omitted, assay buffer was added to make up the volume to 700 µl.

After adding all the constituents, the tubes were gently vortexed for 5-10 seconds and incubated at 4°C for 48 h. At the end of first incubation, 100 µl diluted second antibody (1:25) was added to all the tubes except the total count tubes. The incubation mixture was vortex mixed and incubation continued for an additional 16-18 h at 4°C. After second incubation, all tubes except those for total counts were centrifuged at 1800xg for 40 minutes at 4°C and the pellet counted in a Multigamma counter (LKB-1260).

**Testosterone:** The protocol used for RIA of testosterone is summarized as follows:

<table>
<thead>
<tr>
<th>Set*</th>
<th>Buffer (µl)</th>
<th>Std/QC (µl)</th>
<th>Unknown sample (µl)</th>
<th>Antiserum (µl)</th>
<th>Tracer (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSB (blank)</td>
<td>600</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>Ether blank</td>
<td>500</td>
<td>-</td>
<td>-</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>B0</td>
<td>500</td>
<td>-</td>
<td>-</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Standards/QC</td>
<td>-</td>
<td>500</td>
<td>-</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Unknown samples</td>
<td>-</td>
<td>-</td>
<td>500</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Total counts</td>
<td>500</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>100</td>
</tr>
</tbody>
</table>

* A set comprises of triplicate tubes.

1. After mixing the reagents, incubation was carried out at 4°C for 16 h.
2. Free steroid was separated from antibody bound steroid by addition of dextran coated charcoal suspension at 4°C, vortex mixed and after 15 minutes centrifuged at 3000 RPM for 5 minutes at 4°C.

3. Supernatant containing antibody bound steroid was decanted in a counting glass vial containing 1 ml distilled ethanol.

4. To each vial 8 ml scintillation fluid (4 g PPO and 500 mg POPOP per liter of toluene) was added, equilibrated for 60 minutes at room temperature and counted in a β scintillation counter.

c. **Characterization of antibodies:** Both IF-10 and IF-11 sera were analyzed by:

- Localization studies on sperm by Indirect Immunofluorescence (IIF) and immunocytochemical studies.
- Functional studies by agglutination of normal human sperm and in vitro hamster sperm-oocyte binding assay.
- Pattern of antigenic recognition in western blots.

**Preparation of spermatozoa for indirect immunofluorescence studies:** Freshly ejaculated semen samples were obtained from normal humans by masturbation and bonnet monkeys by electro-ejaculation using a seven inch long copper electrode as a rectal probe. The samples were allowed to liquefy at room temperature and washed twice with phosphate buffered saline (PBS pH 7.4). Sperm from rabbit (New Zealand white), rat (Wistar) and hamster...
(golden Syrian), were obtained from minced cauda epididymides in PBS. The resulting suspensions were filtered through nitex membranes to remove tissue debris. Sperm were sedimented at 600g, washed in PBS and coated as a single layer on slides cleaned with 70% ethanol, air dried and fixed in methanol for 10 minutes and stored in -70°C until use.

**Preparation of spermatozoa for functional studies:** Highly motile human sperm were obtained by swimup procedure. Briefly, freshly ejaculated human semen was allowed to liquefy at room temperature and distributed in aliquots. To these aliquots Biggers Whitten's and Whittingham's media (BWW, Composition/ litre; NaCl 5.54 g, KCl 0.356 g, CaCl₂·2H₂O 0.25 g, KH₂PO₄ 0.162 g, MgSO₄·6H₂O 0.294 g and phenol red (0.5%) 1 ml; Biggers et al. 1971) supplemented with 2mg/ml bicarbonate, 0.4% lactate, 0.01% pyruvate and 3mg/ml human albumin was very carefully layered in a ratio of 1 part semen to 3 parts media.

The tubes were incubated at 37°C for 60 minutes to allow highly motile sperm to swim-up. Spermatozoa which were in the upper 2 ml of the medium were harvested in 3 ml and centrifuged at 600g for 3 min in a clinical centrifuge, resuspended in 1 ml supplemented BWW and counted in a Neubauer's chamber.

**Preparation of samples for pattern of antigenic recognition in western blots**

**Preparation of sperm extracts:** Sperm from human,
monkey, rabbit and hamster washed in PBS (pH 7.4) were extracted with 1% SDS (sodium dodecyl sulfate, Sigma, MO) in the presence of 10mM PMSF (phenyl methyl sulfonylfluoride, Sigma, MO) as protease inhibitor for 2h at room temperature, centrifuged and supernatant stored at -20°C until use.

Preparation of rabbit testicular cytosol: Testes from 5 rabbits (80 weeks) were dissected out, decapsulated and homogenized in 10 ml of 20 mM phosphate buffer (pH 7.4) containing 0.1mM PMSF as protease inhibitor. The homogenate was centrifuged at 1000 x g 3 times followed by ultracentrifugation at 198000 x g for 2h. The supernatant was aliquoted in small volumes and stored at -70°C till use.

i. Localization studies:
**Indirect Immunofluorescence assay:** Immunofluorescence was carried on sperm fixed with methanol as well as human sperm in suspension in PBS. Spermatozoa were incubated in suspension with both sera IF-10 and IF-11 at room temperature for 1 h at a dilution of 1:50 in PBS. Sperm were washed three times in PBS and resuspended in a 1:50 dilution of second antibody - rabbit anti-human conjugated to fluorescein isothiocynate (FITC, Dakopatts, Denmark) at room temperature. After two washes following second antibody incubation, sperm were mounted in PBS containing 10% glycerol and 0.1% p-phenylenediamine (PPD)

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as an antiquencher and observed with a Nikon Optiphot epifluorescence microscope. The slides were processed in the same manner except that washing was done in PBS in a Couplin jar and a humid chamber was used for incubating the slides.

**Immunocytochemical localization:** Testis from a freshly killed 80 week-old male New Zealand white rabbit was dissected out from its surrounding tissues and embedded in a freezing compound ("Tissue-Tek" OCT compound, Miles Inc. Diagnostic division, Elkhart, IN 46515, USA) and sections of 5μm thickness were prepared in a freezing microtome (American Optical) at -21°C onto glass slides cleaned in 70% ethanol. The slides were fixed in methanol for 10 minutes, air dried and stored at -70°C until use. Sections of frozen tissues of rabbit testes were treated with 10% normal goat serum (GIBCO) in PBS for 45 min to block nonspecific binding sites. Sections were incubated with IF-10 and IF-11 at dilution of 1:50 for 1h at room temperature in a humid chamber, followed by three washes with PBS for 5 minutes each. Sections were then incubated with anti-human IgG conjugated to horse radish peroxidase (HRP, Dakopatts, Denmark) at a dilution of 1:200 for 1h at room temperature. After washing, color was developed by incubating the sections with 0.1% di-aminobenzidine in PBS (pH 7.4) containing 0.03% hydrogen peroxide (H₂O₂) (Sternberger 1979). Slides were then washed in PBS and mounted in PBS containing
10% glycerol. Primary antibody was substituted with normal human serum in control experiments.

ii. Functional tests:

Agglutination test: Agglutination was studied using the procedure of Friberg (1974). In order to avoid non specific agglutination, only highly motile sperm devoid of seminal debris and dead cells were used following a swim up (described elsewhere). Sperm concentration was adjusted to $1 \times 10^5$ in 50\(\mu\)l aliquots per well of a 96 well ELISA plate (Linbro, Flow Laboratories, Detroit). Sera IF-10 and IF-11 were diluted from 1:10 through 1:128 serially in PBS after decomplementing at 56°C for 30 minutes in a water bath. Each dilution was separately incubated with an aliquot of sperm and observed at regular intervals of 15 minutes in a Nikon TMS inverted microscope for sperm agglutination. Decomplemented normal human serum was used as a control.

Sperm binding assay:

Preparation of TL-HEPES and TALP: Composition of TL-HEPES is given on the following page. TALP (TL-HEPES supplemented with albumin and pyruvate) was prepared by adding 500\(\mu\)l of 10 mM sodium pyruvate (in 157 mM NaCl stock solution) and 3% bovine serum albumin (BSA) to 50 ml of TL-HEPES.
### Composition of Tyrodes lactate with HEPES (TL-HEPES)

<table>
<thead>
<tr>
<th>COMPONENT</th>
<th>mM</th>
<th>g/100ml</th>
<th>FINAL mM</th>
<th>ml. OF STOCK</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>157</td>
<td>0.92</td>
<td>114</td>
<td>make vol. to 100ml</td>
</tr>
<tr>
<td>KCl</td>
<td>166</td>
<td>1.24</td>
<td>3.16</td>
<td>1.9</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>120</td>
<td>1.76</td>
<td>2.0</td>
<td>1.7</td>
</tr>
<tr>
<td>MgCl₂ . 6H₂O</td>
<td>120</td>
<td>2.44</td>
<td>0.5</td>
<td>0.41</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>167</td>
<td>1.4</td>
<td>2.0</td>
<td>1.2</td>
</tr>
<tr>
<td>Na₂HPO₄*</td>
<td>20.5</td>
<td>0.35</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>295</td>
<td>5.31</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>Na-LACTATE [60% syrup]</td>
<td>150</td>
<td>10.0</td>
<td>6.7</td>
<td></td>
</tr>
<tr>
<td>DDW (millipore)</td>
<td>12.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HEPES</td>
<td>10.0</td>
<td></td>
<td>240mg</td>
<td></td>
</tr>
</tbody>
</table>

* Na₂HPO₄ . 2H₂O is prepared by dissolving 32 mg in 10 ml glucose stock solution (295 mM) of which 1.7 ml is added to TL-HEPES.

Na-Lactate solution: 2.85 ml of 60% Na-Lactate syrup (DL-Lactic acid) is added to 100 ml containing 0.001% phenol red. Before making up the volumes to 100 ml the pH was adjusted to 7.6.

pH of TL-HEPES was adjusted between 7.2 and 7.4 with 400 μl to 600 μl of 1 Normal NaOH.

Preparation of penicillamine, hypotaurine and epinephrine (PHE) solution: PHE solution was prepared as shown below:

1 : Pencillamine - 2 mM: 3 mg/10 ml 157 mM NaCl
2 : Hypotaurine - 1 mM: 1.09 mg/10 ml 157 mM NaCl
3 : Epinephrine - 250 μM

165 mg Na Lactate syrup (60%) and 50mg sodium metabisulfite to 50 ml DDW and pH adjusted to 4.0. To 40 ml of the above solution was added 1.83 mg Epinephrine.

**PHE STOCK SOLUTION (100X)**

- 0.25 ml of solution 1
- 0.25 ml of solution 2
- 0.10 ml of solution 3
- 0.40 ml of 157 mM NaCl stock

PHE stock was divided into 100 μl aliquots and stored at -70°C till use and thawed in ice in dark before use.
Preparation of animals: Twenty-one to twenty-five days old Syrian golden hamsters (Mesocricetus auratus) were superovulated with 40 I. U. pregnant mare serum gonadotrophin (PMSG, Folligon, Intervet International, The Netherlands) and 25 I.U. human chorionic gonadotrophin (hCG, Chorulon, Intervet International, Netherlands) following 52 h of PMSG treatment. Sixteen to eighteen hrs after hCG treatment, oocytes were collected by flushing the oviducts with Tyrodes albumin lactate pyruvate (TALP) (Bavister et al. 1977). The cumuli surrounding the oocytes were dispersed by addition of 0.1% hyaluronidase (Sigma, MO) for 5 minutes. Oocytes were washed three times with TALP medium to remove hyaluronidase and distributed in 50μl drops of media in petri dishes kept in a humid 37°C incubator. Sperm were collected from the cauda epididymides of an adult male of same strain of hamsters and concentration adjusted to 1x10^6 sperm per ml of TALP. These were incubated in the dark at 37°C for 20 minutes in TALP containing 1X PHE [penicillamine, hypotaurine, and epinephrine (Sigma, MO)] as described by Bavister et al. (1977) to allow the sperm to achieve hyperactivated motility. Sperm were then incubated with antisera (IF-10 and IF-11) at 1:50 dilution for 30 minutes. 1x10^5 sperm per ml of antibody treated sperm were then incubated with six oocytes in the same medium at 37°C for 1 hour in duplicate. The loosely attached sperm were removed by repeated passage
through a capillary pipette at the end of incubation. Oocytes were visualized with phase contrast microscopy. In control experiments normal human serum was substituted for IF-10 and IF-11.

iii. Pattern of antigenic recognition:

SDS-polyacrylamide gel electrophoresis (SDS-PAGE): Gel electrophoresis was performed as described by Ornstein (1964) and Davis (1964) and later modified by Laemmli (1970). The recipe for discontinuous SDS separating gel of various percentage of acrylamide is given below:

Recipe for discontinuous SDS separating gels*

<table>
<thead>
<tr>
<th>SOLUTION</th>
<th>7.5%</th>
<th>10%</th>
<th>15%</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Acrylamide: 0.8% bis-Acrylamide</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5M Tris-HCl, pH 8.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DDW</td>
<td>14.5</td>
<td>12</td>
<td>9.5</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>10% Ammonium persulfate</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
</tbody>
</table>

* Volume in ml

Recipe for preparation of stacking gel (3% acrylamide)

<table>
<thead>
<tr>
<th>SOLUTION</th>
<th>ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Acrylamide: 0.8% bis-Acrylamide</td>
<td>1.33</td>
</tr>
<tr>
<td>0.5M Tris-HCl, pH 6.8</td>
<td>2.5</td>
</tr>
<tr>
<td>DDW</td>
<td>6</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.1</td>
</tr>
<tr>
<td>10% Ammonium persulfate</td>
<td>0.075</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.01</td>
</tr>
</tbody>
</table>
Casting of gels: The gel slab was prepared between two glass plates (Studier, 1973) of Bio-Rad mini protean II slab gel electrophoresis apparatus. The thickness of the gel was 1.5 mm equivalent to the thickness of the spacer between the two glass plates. For polymerization of the separating gel, acrylamide solution was poured between the plates up to a height of 5 cm. It was gently overlaid with DDW and polymerized at room temperature for one hour. Before polymerizing the stacking gel, water was removed. The stacking gel solution was poured and a comb of 1.5 mm thickness was inserted and the gel allowed to polymerize for 30-45 min. Tank buffer (0.025 M Tris base, 0.193 M glycine and 0.1% SDS; pH 8.3) was poured into the upper and lower chambers of the electrophoresis apparatus. Care was taken to avoid the trapping of air bubbles below the surface of gel.

Running of gels: Protein samples equivalent to 60μg BSA as estimated by Lowry's method (Lowry et al. 1951) were mixed with an equal volume of sample buffer (composition given below) and heated in a boiling water bath for 3 minutes.

Recipe for preparation of sample buffer

<table>
<thead>
<tr>
<th>SOLUTION</th>
<th>VOLUME USED IN ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Mercaptoethanol</td>
<td>1</td>
</tr>
<tr>
<td>Glycerol</td>
<td>2</td>
</tr>
<tr>
<td>10% SDS</td>
<td>4</td>
</tr>
<tr>
<td>0.5M Tris-HCl, pH 6.8</td>
<td>2.5</td>
</tr>
<tr>
<td>1% Bromophenol blue</td>
<td>0.1</td>
</tr>
<tr>
<td>DDW</td>
<td>0.4</td>
</tr>
</tbody>
</table>
The samples were then loaded into the wells with a 100μl Hamilton syringe (Hamilton MICROLITER #710, Switzerland). Electrophoresis was carried out at 80 Volts till the marker dye reached the other end of the gel.

**Staining of Gels:** Coomassie brilliant blue R-250 (Biorad laboratories, Richmond, CA) was used for staining of gels. A stock solution of 1% stain in DDW was prepared. Gels were stained in 0.125% Commassie blue in 40% methanol and 10% acetic acid for 10 minutes and subsequently destained in a solution of 40% methanol and 10% acetic acid to visualize the protein bands.

**Western blotting:** Western blotting was performed using a modification of the method of Towbin, Staehelin and Gordon (Towbin et al. 1979). After electrophoresis the gels were presoaked in cold transfer buffer (0.025M Tris base, 0.193 M glycine, pH 8.3 and 20% (v/v) methanol) for 30 minutes. The gel was placed on a wet Whatman filter paper and a wet nitrocellulose paper (0.45μm pore size, Biorad Laboratories, Richmond, CA) cut to the size of the gel was then placed on top of the gel. Care was taken to ensure that no air bubbles were trapped between the gel and the nitrocellulose paper. This was followed by another wet Whatman filter paper. The sandwich was placed between blotting fiber pads which were placed in a Biorad plastic casket so that the gel faced the cathode end, and
the paper, the anode. The whole sandwich was lowered into the western blot apparatus (Biorad mini transblot cell, Biorad Laboratories, Richmond, CA) filled with cold transfer buffer so that the gel faced the cathode (Black electrode) and proteins were transferred onto nitrocellulose membrane at 50 volts for 150 minutes at room temperature.

After completion of transfer the nitrocellulose paper was stained in 0.1% (w/v) amido black (Biorad Laboratories, Richmond, CA) in 7% glacial acetic acid solution for 10 seconds and excess stain was subsequently destained in a 7% acetic acid solution to check completion of transfer.

Nitrocellulose membranes were treated with 2% Bovine Serum Albumin (BSA, Sigma, MO) in PBS-Tween 20 (0.05%) for 45 Min. at room temperature to block nonspecific sites. Following washes with PBS-Tween 20, primary antibodies (IF-10 and IF-11) were applied at a dilution of 1:50 for 2h at room temperature. Following washes with PBS-Tween 20 the nitrocellulose was incubated with rabbit anti human IgG conjugated to HRP (Dakopatts, Denmark) at a dilution of 1:300 for 1h at room temperature. Color was developed by incubating the nitrocellulose membranes in solution containing 4-chloronapthol (3.3 mg per ml in methanol) in PBS with 0.01% H₂O₂.
B. Raising of Antisera to Cross-reactive Rabbit Antigens

In order to further characterize the antigen(s) recognized by the infertile sera, polyclonal antisera were raised against the crossreactive 100kDa and 53-55kDa rabbit sperm proteins.

Rabbit cauda sperm proteins were extracted in 1% SDS as described above and run on preparative 10% SDS-PAGE. Protein bands of interest namely the 100kDa and 53-55kDa were identified by aligning stained strips from small slab gels along the edges of unstained gels and sliced out with a scalpel (Hames 1981). Both the 100kDa and 53-55kDa antigens were dissected out using the above procedure with scalpels. Slices containing both the proteins were then separately minced and incubated in 50 mM NH₄HCO₃ containing 0.1% SDS in a ratio of 1ml of gel to 5 ml of NH₄HCO₃. The incubation was carried out at 25°C for 8h. Bands were then centrifuged down and the supernatant lyophilized and redissolved in a smaller volume of DDW. Protein was estimated using the method of Lowry's (Lowry et al. 1951).

1. Animals

Six months old female rabbits (New Zealand white) were obtained from the small animal facility of the National Institute of Immunology for experiments. The animals were bled from the retino-orbital vein using glass capillaries prior to immunizations.
2. Immunization

500 μg equivalent of each protein was separately emulsified with complete Freund's adjuvant (CFA, Sigma, MO) and injected into each rabbit subcutaneously at multiple sites. Boosters were given at regular intervals of 30 days with 250 μg equivalent protein emulsified with incomplete Freund's adjuvant (IFA, Sigma, MO). The titres of antisera to 100kDa and 53-55kDa rabbit sperm proteins were monitored by ELISA (Enzyme linked immunosorbant assay) and IIF to rabbit and human spermatozoa.

3. Characterization of antisera

a. Enzyme Linked Immunosorbant Assay:

Preparation of plates for ELISA: 0.5 μg equivalent protein of rabbit cauda sperm extract in 50 μl of 0.5M carbonate buffer (pH 9.2) was coated per well on 96 microwell plastic plates (Titertek, Flow Laboratories Inc., McLean, Virginia.) and incubated at 37°C for an hour, air dried and stored at -20°C until used.

The plates were incubated with 100 μl/well of 1% polyvinyl alcohol (PVA, Sigma, MO) in DDW for 45 min. at room temperature to block non-specific sites followed by three washes in PBS containing 0.05% Tween 20 for 5 minutes each. The plates were then incubated with anti-100kDa and anti-53-55kDa antisera at a dilution of 1:50 in 50 μl of PBS-Tween 20 three times (0.05%) for 60 min at room temperature. Following washes in PBS-Tween 20 the plates were
incubated with 50μl per well of 1:2000 anti-rabbit IgG-HRP conjugate (Dakopatts, Denmark) for 60 min. Color was developed with 100μl per well of 0.05% orthophenylene diamine (OPD, Sigma, MO) in citrate-phosphate buffer (22.1 mM citric acid and 51.4 mM Na₂HPO₄, pH 5.5) containing 0.01% H₂O₂. The reaction was stopped using 5μl per well of 5N H₂SO₄. The optical density was measured at 490 nM in a 96 microwell reader (Biotek Instruments, Winooski, Vermont).

b. **Indirect immunofluorescence (IIF):** IIF was done on methanol fixed rabbit and human spermatozoa as described previously. Anti-rabbit IgG conjugated to FITC was used as a second antibody at a dilution of 1:200.

c. **Western blotting:** Fifty micrograms equivalent protein per well of human SDS sperm extracts were run on 10% SDS-PAGE and transferred on to a nitrocellulose sheet as described previously.

The nitrocellulose sheets were blocked and washed as described previously. Following washing in PBS-Tween 20, the sheets were incubated with anti-100kDa and anti-53-55 kDa antisera at a dilution of 1:100 in PBS-Tween20 for 60 min. at room temperature, washed in PBS-Tween20 three times and incubated for 60 minutes with anti-rabbit IgG-HRP conjugate (Kirkegaard & Perry Laboratories Inc., Maryland) at a dilution of 1:500 at room temperature.
Color was developed using 4-α chloronapthol in methanol and \( \text{H}_2\text{O}_2 \) as described above.

d. **Agglutination**: Agglutination test was done as described above with human sperm swimup with the polyclonal antisera up to a dilution of 1:128.

### II. EFFICACY STUDIES WITH RELEVANT ANTIGENS

#### A. Immunizations

1. **Animals**

   Sixteen weeks old female "Wistar" rats of proven fertility were used for active immunization studies of the relevant antigens from the rabbit testicular cytosol to check their efficacy *in vivo*. Rats were chosen for efficacy studies due to the following advantages:

   1. The antisera under study crossreacted with the acrosome of rat sperm and therefore rat was one of the cross reactive species.
   2. Cyclicity patterns of the species are known and clearly demarcated compared to hamsters and rabbits which were the other crossreactive species.
   3. Rats are comparatively easier to handle and a large number of animals could be included in a single group.

   Prior to immunization the cyclicity of these animals were monitored for six cycles. Those animals that showed regular cyclicity patterns during this period were used for the study.
The animals were bled (from the retino-orbital vein using glass capillaries) twice at weekly intervals prior to immunization.

Rats were divided into two groups of twenty each, one for the 53kDa antigen(s) and the other for the 27kDa antigen(s). A separate group of fifteen animals consisted of controls which received only the adjuvant.

2. Preparation of immunogen

Both 53kDa and 27kDa proteins from rabbit testicular cytosol were eluted from preparative SDS-PAGE and subsequently concentrated by lyophilization. Protein was estimated (Lowry et al. 1951) using BSA as the standard.

One milligram each of the 53kDa and 27kDa protein was mixed separately with one ml of alum (3% AlOH\textsubscript{3}, Trade name 'SUPERFOS', Speciality Chemicals a/s, Vedbaek, Denmark) and adsorbed at 25°C for 2h making the final concentration to 50μg protein per 50μl alum per rat to which 100μg Nor-MDP [3-O-D-glycollyl-(3-deoxy-D-GlcNac)-L-Ala-D-IsoGln] was added as the adjuvant. Nor-MDP is a derivative of muramyldipeptide which is an ubiquitous constituent of bacterial cell wall. MDP is basically MurNac-L-Alanyl-D-Isoglutamine, the smallest adjuvant active compound from bacterial cell wall peptidoglycans. MDP was first synthesized by Merser et al. (1975). MDP and its derivatives can stimulate antibody responses to a variety of natural and synthetic antigens. It increases the levels of circulating antibodies and induces delayed
type hypersensitivity to protein antigens (Ellouz et al. 1974; Kotani et al. 1975b; Merser et al. 1975). Moreover, as a constituent of CFA, MDP is more active than mycobacteria on a weight basis. It further appears that this series of simple compounds also induces adjuvant related as well as pharmacological effects. For control group similar schedule was followed as above with saline substituting for the protein.

3. Route and schedule

Rats were immunized on the femur muscle with the prepared immunogen using a 26 1/2 'G' needle. Three primary injections were given at an interval of three weeks and a booster on day 84. Dose of the antigen was 50 μg/rat for first injection and 25 μg for all subsequent injections.

B. Characterization of Antisera

1. Antibody titers

Antibody titers in the rat sera were monitored by IIF assay on rabbit, rat and human sperm and by ELISA with the respective antigens.

a. IIF index: Human sperm ejaculates were allowed to liquefy at room temperature and were then washed twice with PBS (0.05M, pH 7.4). Rat and rabbit sperm were obtained from minced cauda epididymides by flushing them out in PBS. The resulting sperm suspensions were filtered
through nitex membranes to remove tissue debris, washed in PBS and were then coated as a single layer on glass slides cleaned with 70% ethanol, air dried and fixed in methanol for 10 min. IIF was performed by incubating the slides with 1:50 and 1:100 dilutions of the test antisera collected from immunized rats for 1h at room temperature. Following two washes in PBS, incubation was performed with anti-rat IgG conjugated to FITC at a dilution of 1:100 for 30 min. Slides were washed and mounted in 10% glycerol containing 0.1% p-phenylenediamine (PPD) as an antiquencher. The dilutions at which immunofluorescence was visible were taken as the IIF index.

b. ELISA: ELISA was done as described previously in Section I of Materials & Methods. Briefly, 0.5μg equivalent protein of either the 53kDa or 27kDa antigens in 50μl of carbonate buffer (pH 9.2) were coated per well on 96 microwell plastic plates and incubated at 37°C for an hour, air dried and stored at -20°C until used.

Plates were blocked with 100μl/well of 1% polyvinyl alcohol in DDW for 45 min. at room temperature followed by three washes in PBS containing 0.05% Tween 20 for 5 min each. The respective plates were then incubated with anti-53kDa and anti-27kDa rat antisera at a dilution of 1:100 in 50μl of PBS-Tween 20 thrice (0.05%) for 60 min at room temperature. Following washes in PBS-Tween 20, plates were incubated with 50μl per well of 1:3000 anti-rat IgG-HRP conjugate (Dakopatts, Denmark) for 60 min.
Color was developed with 100µl per well of 0.05% OPD in citrate-phosphate buffer (pH 5.5) containing 0.01% H₂O₂. The reaction was stopped using 5µl per well of 5N H₂SO₄. Optical density was measured at 490 nM.

2. SDS-PAGE and western blotting

Rabbit testicular cytosol was fractionated by SDS-PAGE as described earlier and the proteins transferred onto nitrocellulose membranes in a Biorad transblot apparatus at room temperature for 3h (Refer Section I of Materials & Methods). Blots were treated with 2% BSA (Sigma, MO) in PBS -Tween (0.05%) for 45 minutes at room temperature to block non-specific sites. Rat sera at 1:100 dilution from immunized animals were applied as the primary antiserum for 2h at room temperature followed by three washes with PBS-Tween (0.05%). Anti rat IgG conjugated to horse radish peroxidase (Cappel, USA) was applied at a dilution of 1:500 as the second antibody for 1h at room temperature followed by three washes in PBS. Color was developed by incubating the nitrocellulose strips in a solution containing 4α-chloronapthol (3.3 mg per ml methanol) in PBS with 0.01% hydrogen peroxide.

C. Mating Studies

Each immunized female rat was placed with an adult male "Wistar" rat of proven fertility. Vaginal smears were examined every morning to check cyclicity and for the presence of sperm. The presence of sperm in the smear
was taken as an indication of mating. The males were rotated amongst the immunized females once on every eighth day to increase the likelihood of successful mating.

III. CHARACTERIZATION OF RELEVANT ANTIGENS IDENTIFIED BY INFERTILE SERA

A. Preparation of Antigens from Rabbit Testicular Cytosol

Rabbit testicular cytosol was prepared as described previously in Section I of Materials & Methods.

1. Preparative polyacrylamide gels

Samples containing rabbit testicular cytosol were mixed with equal volume of sample buffer (62 mM Tris-HCl pH 6.8, 2% SDS, 5% mercaptoethanol, 10% glycerol and 0.001% bromophenol blue) and heated in a water bath at 95°C for 3 min. 200 µg protein of rabbit testicular cytosol was loaded into each well. Electrophoresis was carried out on 10% polyacrylamide gels as described in Section I of Materials & Methods. The gels were run at 80 Volts for 2h, stained in 0.125% Coomassie blue R-250 in 40% methanol and 10% acetic acid for 10 min and subsequently destained in 40% ethanol and 10% acetic acid to visualize the protein bands.

2. Elution of relevant proteins from gels

Protein bands of interest namely the 53kDa and the 27kDa were identified by aligning stained strips from
small slab gels along the edges of unstained gels and ... sliced out with a scalpel (Hames 1981). Both the 53kDa and 27kDa antigens were dissected out using the above procedure with scalpels. Slices containing both the proteins were then separately minced and incubated in 50 mM NH₄HCO₃ containing 0.1% SDS in a ratio of 1 ml of gel to 5 ml of NH₄HCO₃. The incubation was carried out at 25°C for 8h. Bands were then centrifuged down and the supernatant lyophilized and redissolved in a smaller volume of DDW. Protein was estimated using Lowry's method (Lowry et al. 1951) using BSA as standard. The estimated protein was aliquoted and stored at -20°C until use.

B. Characterization of Proteins

1. Lectin binding studies

Rabbit testicular cytosol, the 53kDa proteins and the 27kDa proteins were electrophoresed and transferred onto nitrocellulose paper. The nitrocellulose strips were blocked in PBS containing 10 mM Tris and 0.5% Tween 20 for 60 minutes followed by washing in PBS-Tris. Concanavalin A (10μg/ml) in PBS-Tris Tween (0.05%) was used as a probe during primary incubations to detect lectin binding sites of these proteins. Secondary incubations were carried out with horseradish peroxidase [100μg/mL]. Reactions were visualized by incubation of the blot with 0.3% 4a-Chloronapthol containing 0.01% H₂O₂ (Cheng et al. 1985)
2. Deglycosylation studies

a. Chemical deglycosylation: Chemical deglycosylation was performed with whole rabbit testicular cytosol according to the procedure of Cheng and co-workers (1985) using trifluoromethane sulfonic acid (TFMS) as deglycosylating agent. Rabbit testicular cytosol was lyophilized in 1 mg aliquots in glass vials. Each vial was treated with 450μg of TFMS and 50μl of anisole in a nitrogen atmosphere. Reaction was carried out at 25°C for 6h. Proteins were precipitated by using an ether pyridine mixture in a ratio of 9:1 respectively. Precipitates were extensively dialyzed and resuspended 0.1M NH₄HCO₃ and extensively dialyzed against same solution and resuspended in buffer containing 20 mM Tris (pH 7.4).

b. Enzymatic deglycosylation: Enzymatic deglycosylation was carried out using β-galactosidase, α-mannosidase, neuraminidase and β-N-acetyl-glucosaminidase (Sigma, MO) as deglycosylating agents (Montreuil et al. 1986). The 53kDa and the 27kDa proteins eluted from gels were separately lyophilized in 50μg aliquots in eppendorf tubes. Proteins were dissolved in 50 mM acetate buffer at appropriate pH of the enzymes (pH 4.2 for α-mannosidase, pH 4.4 for neuraminidase and pH 5.0 for β-N-acetyl-glucosaminidase). 20mM sodium phosphate supplemented with 10 mM acetic acid was used for β-galactosidase instead of acetate buffer. Reaction mixture with mannosidase was
supplemented with Zn\(^{++}\) salts at 2 mM concentrations to stabilize the enzyme towards a stable temperature and pH (Montreuil et al. 1986). PMSF (10 mM) was added to safeguard against protease activities in enzyme preparations in all reactions. The reaction was carried out at 37°C for 24h after adding 0.125 units of the enzyme to each aliquot.

The protein samples deglycosylated by chemical and enzymatic methods were mixed with equal volume of sample buffer and run on 10% SDS polyacrylamide gels. Crude untreated rabbit testicular cytosol was used as a control for samples treated with TFMS. The 53kDa and 27kDa native proteins were used as the respective controls for enzymatic deglycosylation studies.

The treated and native proteins run on SDS-PAGE, were transferred onto nitrocellulose as described previously and incubated with anti-53kDa and anti-27kDa antisera. Concanavalin A was used as a marker to indicate complete deglycosylation of the respective proteins for TFMS and α-mannosidase treated preparations.

3. Isoelectric focusing

Isoelectric focusing was carried out on slab gel electrophoresis apparatus using carrier ampholytes (pI 3.5-10 and pI 4-7, Pharmacia, Sweden) in the presence of urea (Giulian et al. 1983). The composition of the urea gel used for IEF is shown below:
<table>
<thead>
<tr>
<th>COMPONENT</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDW</td>
<td>7.5 ml</td>
</tr>
<tr>
<td>UREA</td>
<td>10.2 g</td>
</tr>
<tr>
<td>AMPHOLYTES (pI 3-10)</td>
<td>0.7 ml</td>
</tr>
<tr>
<td>AMPHOLYTES (pI 4-7)</td>
<td>0.187 ml</td>
</tr>
<tr>
<td>30% ACRYLAMIDE</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>NONIDET P40</td>
<td>0.375 ml</td>
</tr>
<tr>
<td>AMMONIUM PERSULFATE</td>
<td>0.087 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.037 ml</td>
</tr>
</tbody>
</table>

**Preparation of sample:** Fifty μg of 27kDa and 53kDa proteins were separately lyophilized in eppendorf tubes. The lyophilized protein was treated with sample buffer (75 μl 3.5-10 pH carrier ampholyte, 390 μl DDW and 18% glycerol).

Polyacrylamide containing urea in the presence of a mixture of ampholites of pH 3.5-10 and 4-6 was used to separate the proteins. Anolyte was 0.02M acetic acid and catholyte was 0.02M NaOH. Twenty-five μg of 27kDa and 53kDa each was loaded on to each lane. Standard pI markers were also loaded on parallel lanes to facilitate calculation of the pI of various isoelectric species of the proteins.

Electrophoresis was carried out for 18h at 150V. Gels were either incubated in transfer buffer for 20 minutes before being put to transfer or fixed in 20% trichloroacetic acid (TCA) for 10 minutes followed by fixation in 40% ethanol and 10% acetic acid containing 0.25% SDS for 30 min. This was followed by brief washing with solution containing 40% ethanol and 10% acetic acid for 30 min. Gels were subsequently stained with 0.125%
coomassie blue G-250 in 40% ethanol and 10% acetic acid for 20 min. The gels were subsequently destained in 40% ethanol and 10% acetic acid solution.

The isoelectric species transferred onto nitrocellulose were processed for antibody screening with anti-53kDa antisera and anti-27kDa antisera.