Classification of male factor infertility relevant to in-vitro fertilization insemination strategies using mannose ligands, acrosome status and anti-cytoskeletal antibodies

Susan Benoff1,2, Michele Barcia1, Ian R.Hurley3, George W.Coopera, Francine S.Mandel3, Susan Heynen3, William T.Garside1, Bruce R.Gilbert3 and Avner Herschlag1

1Department of Obstetrics and Gynecology, North Shore University Hospital, Cornell University Medical College, 300 Community Drive, Box-Math-Biomedical Science Research Center Room 125, Manhasset, New York 11030. 2Center for Environmental Science, College of Staten Island, City University of New York, Staten Island, New York. 3Department of Research, North Shore University Hospital and Department of Public Health, Cornell University Medical College, 3Department of Obstetrics and Gynecology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, and 4Department of Surgery, North Shore University Hospital-Cornell University Medical College, Manhasset, New York, USA.

Polyvalent mannose ligands in the presence of free mannose act as zona pelucida agonists which rapidly induce acro-
some exocytosis in competent motile human sperm from fertile donors following in-vitro capacitation. Quantification of the binding patterns of fluorescein isofoxynoate-labeled mannosylated albumins and of specific anti sera which recognize mannose receptors and other related integ-
ral sperm membrane proteins as well as the incidence of induced acrosome exocytosis after capacitation has allowed us to identify three categories of male infertility. Category 1 males have normozoospermic semen parameters, their spermatozoa have elevated sperm cholesterol values and fail to fertilize oocytes in vitro after standard short-term incubations. These spermatozoa do not bind mannose ligands and do not show spontaneous or induced acrosome reactions, but treatments to remove cholesterol from the spermatozoa (e.g. prolonged incubation in the presence of steroid acceptors) confer the ability to fertilize. Cholesterol loaing and unloading experiments have demonstrated the reversible character of sperm membrane properties in category 1 male infertility. Category 2 males have normal appearing spermatozoa in semen which express mannose ligand receptors on incubation, but fail to undergo acro-
some reactions in response to mannose treatment. Interest-
tingly, all category 2 males identified in this study have clinical varicoceles. Category 3 males have semen which may be normozoospermic or teratozoospermic with, in some cases, high percentages of tapering spermatozoa in the absence of clinical varicocele. Spermatozoa from category 3 men are deficient in a superfertility of integral membrane proteins whose cytosplastic tails have myosin motors as identified by amino acid sequence analysis and anti-myosin antibody reactivity. Their spermatozoa do not express mannose fagand receptors or undergo induced acrosome reactions. Fertilization with category 2 and 3 semen is not achieved by microinulation procedures. These findings illustrate the practical application of basic research for infertility classification.

Key words: acrosome status/cytoskeletal signal transduction/male infertility/mannose lectin/membrane cholesterol

Introduction

A male factor is thought to contribute to reproductive failure in at least 40% of couples presenting for fertility evaluation (Oehninger et al., 1988; Gilbert et al., 1994). Physical (e.g. anatomical or neuroendocrine defects, scrotal trauma anti-sperm immunity, accessory gland dysfunction, genetic factors, diabetes, hypertension), infectious (e.g. childhood diseases, systemic illnesses, venereal disease) and environmental (e.g. alcohol consumption, cigarette smoking, medications, occupa-
tion) causes must be considered and can provide guidance for treatment in cases where male subfertility is associated with sexual dysfunction and/or with oligo- or azoospermia (Burgos-Olmedo et al., 1990; Mattison et al., 1990; Schaefer and Knauer, 1993; Gilbert et al., 1994; Howards, 1995).

Traditional diagnostic and treatment methods are, however, of limited use to the andrologist or practising urologist when the standard parameters of semen analysis are considered to be normal (Oehninger et al., 1988; McKenna et al., 1992). Clinical evaluation of male fertility has recently focused on several morphological and functional parameters which directly relate to fertilization in vitro and in vivo. Among these, the ability of human spermatozoa to undergo spontaneous and induced acrosome reactions after incubation in vitro is critical for fertilization, since only acrosome-reacted human spermato-
zoa penetrate through the human zona pelucida (Liu and Baker, 1993). Both the cumulus oophorus and the zona pelucida play a role in the selection of those sperm with normal oral head forms which may ultimately undergo an acrosome reaction at the zona surface (Carerell et al., 1995; Liu and Baker, 1994a). As a result, we (Benoff et al., 1995c) and others (Kreger et al., 1986; 1988; Oehninger et al., 1988; Fiorentino et al., 1994; Ombelet et al., 1994; Oguz et al., 1994; Parinaud et al., 1995) have sought to define insemination conditions based on sperm morphology and acrosome status which would predict and/or optimize fertilization in vitro. For example, we have demonstrated that, even when defects in acrosome morphology can be detected by inspection, e.g. when >50% of spermatozoa had small acrosomes, it is still possible
to achieve fertilization and pregnancy rates equivalent to those obtained with normal semen simply by inseminating with high total numbers of spermatozoa, thereby increasing the number of normal-appearing spermatozoa per oocyte. Nevertheless, despite the utilization of these high insemination doses, fertilization failure is still observed in a small percentage of normozoospermic cases and can often be attributed to specific deficits in sperm function (Benett et al., 1993b,c, 1994b, 1995b).

To investigate the reasons underlying fertilization failures, we have performed a retrospective comparison of semen specimens that have fertilized oocytes in in-vitro fertilization (IVF) with those that failed to fertilize. Three different assays were performed. Previously, we quantified the zona pellica adhesion potential of motile sperm populations using a neoglyco- protein ligand, fluorescein (FITC)-conjugated mannoseylated bovine serum albumin (BSA). The percentage increase in motile spermatozoa capable of binding this probe over their heads following exposure to capacitating conditions correlates with successful fertilization in IVF (Benett et al., 1993b,c).

In the current studies, we first compared mannose receptor expression by patient and fertile donor spermatozoa before and after a standard 18 h incubation at 37°C and/or after 3–5 days incubation at room temperature.

Only a subpopulation of motile human spermatozoa are ever able to undergo an acrosome reaction (Benett et al., 1995c,e). Motile spermatozoa which can be induced to acrosome react in response to appropriate stimuli represent the subpopulation capable of undergoing an acrosome reaction at the zona surface (Henkel et al., 1993). Therefore, secondly, we also examined the ability of acrosome-intact sperm, expressing head-directed mannose binding sites, to undergo an acrosome reaction under conditions which mimic those that spermatozoa would encounter by binding to authentic zonae, e.g., following exposure to polyvalent mannose ligands = N-mannose monocar-
diyl (Benett et al., 1995e). We compared the percentages of induced acrosome loss obtained by this protocol with that stimulated by progesterone or by ionomycin, a calcium ionophore.

Our third assay is based on previous demonstrations that:

(i) in motile sperm populations from fertile donors exposed to physiological agonists which stimulate acrosome loss, the directed movement of mannose binding sites occurs from the head plasma membrane overlying the acrosome cap to that over the equatorial/post-acrosomal segment (Bettelf et al., 1995e), and that (ii) the human sperm surface mannose receptor is a member of a superfamily of integral sperm membrane proteins which display antigenic and amino acid sequence homologies with the globular head of myosin heavy chains in skeletal and cardiac muscle (Bettelf et al., 1994a). The third assay is also based on data, appearing herein, that the topo-

graphical distribution of all spermatozoa unconventional myosin-like proteins correlates with the state of the acrosome.

We therefore postulate that the induction of the human sperm acrosome reaction is initiated by the interaction of the surface receptors' myosin-like molecular motors with the actin/cytoskeleton cytoskeleton underlying the sperm head plasma membrane. To test this hypothesis in cases where an acrosome reaction insufficiency was noted, we compared the level of anti-myosin antibody-reactive protein expression in motile spermatozoa from patients with infertility by Western blot analysis of whole sperm protein extracts.

Three categories of abnormalities in maionzae receptor expression were identified. The results of these studies help to explain reproductive failure in cases with 'occult' male factor infertility and provide information concerning the potential for future reproductive success. We suggest that useful IVF- assisted fertilization strategies could be devised based on our classi-

fication of sperm plasma membrane deficits.

Materials and methods

Media and chemicals

Modified Ham’s F-10 medium (Formula No. 90-8050PC) was obtained from Gibco Laboratories (Grand Island, New York, USA). Unless otherwise noted, all reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Human semen specimens

All protocols employing human subjects were reviewed and approved by the Institutional Review Board of North Shore University Hospital, New York, USA.

Donors of known fertility participated after giving written informed consent. The semen parameters of specimens from these donors were within the normal ranges for morphology, motility and number based on world Health Organization (WHO, 1995) criteria.

Portions of semen specimens produced for diagnostic purposes were obtained from patients from university-based IVF-

assisted reproductive technology programmes and from a male stylogy private practice. Spermatozoa positive for immunoglobulin (IgA and IgG anti-sperm antibodies (ASAs), detected by direct immunofluorescent binding (Bronson et al., 1982), were specifically excluded from this study because ASAs inhibit membrane cholesterol efflux and the concomitant membrane fluidity changes needed for surface expression of mannose-ligand receptors (Bronte et al., 1995a). Semen which fertilized <70% of retrieved metaphase II oocytes were chosen at random from among specimens with normozoospermic parameters. In the 27 cases of failed fertilization in IVF ultimately chosen for study, confounding ovarian factors could be excluded. The medical history of 20 of the male partners was unsatisfactory; the remaining seven had varicocele. Irrespective of the presence or absence of varicocele, the studied males would not have been classified as 'male factor' by the strict morphological criteria advocated for IVF (Kanfer et al., 1986, 1988; Grow et al., 1994; Dumbell et al., 1994) as >50% of their spermatozoa had normal morphology. Immunocyto cytometry was not required for these unidentified specimens.

Semen preparation for experimental studies

Fresh semen specimens, collected by masturbation after 3–5 days of abstinence, were subjected to routine semen analysis (WHO, 1992). Spermatozoa from fertile donors and from some of the pathological practice patients were then selected for motility by a 'swing-up' method as described previously (Bronson et al., 1982). The remainder of the motile population analyzed were obtained from semen frozen in N-trichydroxysuccinyl-2-aminoethyl-sulfonamido acid (hydroxyethyle-lysine-citrates) (TEST)-yolk-buffered medium con-
taining glycerol (Irvin Scientific, Sama Ana, CA, USA). In these cases, three to 20 million gradient-fractions, each 70–80% of the starting volume, were used.

1906
(1,1,1) and centrifuged for 55 min at 300 g. Prior comparison of
zacheted freshly ejaculated and frozen/thawed semen from fertile
donors and from infertility patients has demonstrated that mannone
binding, acrosome status and membrane cholesterol characteristics
are unaltered by liquid nitrogen storage (Benoff et al., 1993a).

Untrated (‘fresh’ or unacclimated) spermatozoa isolated by swim-
up or Percoll gradient centrifugation were prepared for analysis by
centrifugation (500 g for 8 min) to concentrate the spermatozoa. To
induce capacitation, spermatozoa were pelleted, resuspended in Ham’s
F-10 containing 30 mg/ml charcoal/diluted (Chen, 1967) human
serum albumin (HSA) at a density of 12×10^6 cells/ml and incubated
for 16-20 h at 37°C in 5% CO_2 in air or for 2-3 days at room
temperature. In some experiments HSA was replaced with 5 mg/ml
BSA. At the end of incubation, spermatozoa were collected by
centrifugetion and their motility was assessed by phase-contrast
microscopy.

**Visualization of α-mannose binding sites with fluorescein-conjugated
mannosylated bovine serum albumin**

Motile sperm populations were surface-labelled with 100 μg/ml
fluorescein isothiocyanate (FITC)-conjugated mannosylated BSA
(Man-FITC-BSA; Sigma No. A1730) in a Ca^{2+}-supplemented
buffer as previously described (Benoff et al., 1993b). Control
reactions contained 100 μg/ml FITC-conjugated BSA. Motility and
viability (by eosin Y dye exclusion) were assessed at the beginning and
end of the labelling protocol.

Man-FITC-BSA binding by viable spermatozoa was enumerated as
whole head plus midpiece (pattern II) or equatorial/post-equatorial
regions plus midpiece (pattern III) (Figure 1A,B; Benoff et al.,
1993b,d, 1994b). Coded slides from each specimen were examined
×400 magnification by two observers, each scoring at least 300
spermatozoa in 10-20 microscopic fields, with 5-7% variation in
scoring between observers.

**Evaluation of acrosome status**

Acrosome-intact and acrosome-reacted Man-FITC-BSA labelled sper-
matids were also penta-esterified and differentiated by subsequent
reactions with 100 μg/ml rhodamine-labeled Photovue arginine
(RIC-PSA; Vector Laboratories Inc., Burlingham, CA, USA) in
diatized water as described by Cross et al. (1986). Sperm were scored as:
(i) acrosome-intact if the anterior and equatorial regions of the
head were uniformly RITC-PSA labelled; or as (ii) acrosome-reacted
if only the equatorial segment was labelled or if sperm heads were
completely RITC-PSA negative (Figure 1C). At least 300 spermatozoa
in a minimum of 20 microscopic fields were scored for Man-FITC-
BSA binding and acrosomal status by successive adjustments of the
barrier and excitation filters.

**Induction of the acrosome reaction**

Three protocols were employed in order to study the potential of
motile human sperm populations to undergo an induced acrosome reaction:
(i) to examine the effect of potentiation reagents on acrosome status, 75 mM α-mannose monosaccharide was included in the
calcium-supplemented buffer as described above during per-
wash with Man-FITC-BSA surface labelling reactions (Benoff et al.,
1996a); (ii) to examine the effect of cycloheximide on acrosome status, spermatozoa were incubated for 20 min at 37°C in
calcium-supplemented control buffer containing 1 μg/ml procaspase
(Sigma No. P0130); control reactions were performed in the absence
of procaspase as well as in the presence of 0.1% ethanol (the
vehicle used to prepare the initial concentrated procaspase stock
solution) (Benoff et al., 1995a); (iii) to examine the effect of a non-
physiological stimulus on acrosome status, spermatozoa were exposed

---

*Figure 1. Phallomicrographs taken with phase-contrast and with two UV-epifluorescence fluorimemts of capacitated human spermatozoa double-labelled with Min-fluorescein isothiocyanate (FITC)-bovine serum albumin (BSA) and rhodamine-labelled Photovue arginine (RITC-PSA). (A) Phase-contrast image of two spermatozoa. (B) The large arrow indicates a spermatozoon labelled with Man-FITC-BSA in pattern II whereas the small arrow denotes a spermatozoan labelled in pattern III. (C) The corresponding RITC-PSA labelling patterns demonstrate that the spermatozon exhibiting pattern II Man-FITC-BSA binding is acrosome-intact (large arrow). The spermatozoon labelled with Man-FITC-BSA in pattern III had undergone an acrosome reaction (small arrow). us. 3 μm isorosmyin following the protocol of Thomas and Meizel (1988). Control aliquots were exposed to 0.2% ethanol, the solvent
used to prepare the isorosmyin stock solution. Alternative conditions for capacitating incubations Three procedures were used to strip the spermatozoa of any surface-
bound inhibitory coating protein, possibly originating in the seminal
plasma: (i) fresh semen was subjected to Percoll density gradient
centrifugation (Gilbert et al., 1991); (ii) swim-up sperm were treated
for 30 min at room temperature with NaCl in Ham’s F-10-containing
albumin (toxic strength = 390 mOsm) (O’Korth, 1976). Following
these two treatments, spermatozoa were washed three times with
Ham’s F-10, resuspended in Ham’s F-10 supplemented with 30 mg/ml
HSA and incubated as described above; (iii) swim-up spermatozoa
were diluted 1:1 with TESTeal buffer and incubated for 16-20 h
at 37°C (Bolanos et al., 1983; Johnson et al., 1983; Carroll et al., 1992).
In order to increase the proportion of spermatozoa undergoing the
acrosome reaction, motile spermatozoa were incubated in Ham’s F-
1907
Determination of sperm cholesterol concentration

Relative concentrations of sperm plasma membrane-associated, non-esterified cholesterol were determined by gas-liquid chromatography on an Rtx-50 column (Restek Corp., Bellefonte, PA, USA) as previously described (Benoff et al., 1995d). Prior observations indicate that neither the total sperm sterol content nor the incremental change in membrane cholesterol content following overnight incubation in albumin-supplemented medium varies significantly between independent ejaculates from the same male (Benoff et al., 1993c,d).

Cholesterol loading and unloading of the sperm plasma membrane

To increase the plasma membrane content of non-esterified cholesterol 2 to 5-fold, swim-up spermatozoa were incubated at room temperature for 16-20 h in Ham's F-10 containing 1 mg/ml cholesterol-saturated BSA (C-BSA) (Benoff et al., 1995a). To then decrease membrane non-esterified cholesterol content, C-BSA-incubated spermatozoa were washed three times with Ham's F-10 and subsequently subjected to capacitating incubations in the presence of 30 mg/ml dilauroyl HSA as described above.

Indirect immunofluorescence cytchemistry

Spermatozoa were washed three times with PBS and smeared onto the well of a precleaned heavy Teflon-coated (RTC) microscope slide (#1170, Cell-Line Associates, Inc., Newfield, NJ, USA), and allowed to air dry. The buffer system employed for immunohistochemical labelling was adapted from Dano and Fischman (1984) and includes 0.5% Triton X-100 to effect cell permeabilization and allow primary antibody access to possible internal antigens. Rabbit polyclonal sera against smooth and skeletal muscle myosin (Sigma No. M-7678) served as primary antibody; preimmune rabbit serum was employed as control. Spermatozoa were reacted sequentially with primary antibody and human serum protein preabsorbed, fluorescein-conjugated sheep anti-rabbit IgG for 1 h each at room temperature. Specimens were viewed at ×600. The proportion of spermatozoa showing different topographical patterns (see Results section and Figure 6, middle panel) of labelling were assessed by inspection of mounted slides stored at 4°C for <2 weeks before analysis.

To correlate the topography and the percentage of sperm binding anti-cytoplasmic protein antibodies with acrosomal status, spermatozoa were double-labelled with RTC-TPA (Figure 6, lower panel). Spermatozoa were scored visually, following successive adjustments of the barrier and excitation filters. Coded slides from each specimen were examined by two observers, each scoring at least 300 spermatozoa. Where required for documentation, identical fields were photographed on Kodak TMAX film using both phase-contrast and epifluorescence illumination. All photographs were developed for the same length of time at 68°F and printed with identical exposure times.

Western blot analysis

Whole sperm proteins were extracted by non-ionic detergent into a buffer containing a cocktail of protease inhibitors, size fractionated and transferred to membrane filters as previously described (Benoff et al., 1995c). Transferred proteins were probed with the same anti-myosin antibodies employed in the immunocytochemical labelling reaction. Control transfers were probed with pre-immune rabbit serum. Membrane-bound antibody was detected by colour reaction with a complex containing secondary antibody biotin-avidin bridged to alkaline phosphatase (Benoff et al., 1995c). For the purposes of documentation, stained membranes were immediately photographed with Polaroid 667 film.

Statistical analyses

All statistical analyses were performed with the SAS/PC software package (SAS Institute Inc., Cary, NC, USA). Statistical significance was set at P < 0.05.

Results

Category 1: infertility associated with elevated concentrations of non-esterified cholesterol in the sperm plasma membrane

The first category of patients with male factor infertility exhibited zona binding failure in IVF despite normozoospermic semen parameters. Motile sperm populations obtained from these patients had a deficiency in their ability to undergo capitation during standard incubations in Ham's F-10 + 30 mg/ml HSA for 18 h at 37°C. Typical findings for such motile populations (n = 11) and comparisons with spermatozoa from fertile donors (n = 25) are shown in Figures 2 and 3.

Only low percentages of motile spermatozoa in these patients exhibit head-directed Man-FITC-BSA binding. The values for incubated acrosome-intact spermatozoa expressing surface mannose ligand receptors were significantly below the 20% head-directed Man-FITC-BSA binding which characterizes fertile sperm populations under capacitating conditions (Figure 2). In addition, only low percentages of untreated or incubated motile spermatozoa were observed to have undergone a spontaneous acrosome reaction (Figure 3). This is in contrast to fertile donors, wherein an increase in acrosome loss is observed in association with capacitation (Figure 3). As predicted, incubated spermatozoa from category 1 patients fail to acquire the ability to undergo an acrosome reaction stimulated by polyvalent mannose ligands + mannose monosaccharide (Figure 3).

The most striking feature of category 1 men was the sterol composition of their sperm plasma membrane (Figure 4C, typical result). Abnormally high concentrations of non-esterified cholesterol in these spermatozoa were detected. Before and after 18 h incubation under capacitating conditions, the value for relative free cholesterol per cell was >2.0. We have previously demonstrated that the relative free cholesterol content of fertile donor spermatozoa ranges from 0.14-0.174% per cell in untreated specimens and decreases to 0.11-0.13% in duplicate aliquots of the same specimens exposed to capacitating conditions (Benoff et al., 1993c) with concomitant translocation of mannose receptors from subplasmalemmal stores to the sperm surface. Therefore these data suggest a defect in membrane fluidity as the underlying cause of infertility in category 1 males.

To confirm this interpretation, three sets of experiments were performed. In the first series, motile sperm populations from patients were split into a maximum of five parts. Four treatment regimens were applied to increase the penetration of zona-free hamster oocytes by human spermatozoa. In order to provide their ability to promote acrosome loss and also to increase surface expression of mannosidase receptors in comparison with standard swim-up and incubation in Ham's F-10 + 30 mg/ml HSA overnight at 37°C in 5% CO2/95% air (Figures 4A and 4B, typical results). None of these treatments increased the percentage of spermatozoa exhibiting Man-FITC-BSA binding and/or the percentage of spermatozoa exhibiting spontaneous 1908
Figure 2. A repeated measures analysis of variance (RMANOVA) demonstrated that significant differences exist in mean response expression between fertile females and males that failed to fertilize oocytes in IVF (P < 0.0001). These differences were further analyzed by pairwise contrasts (paired t-test) and a minimum of a two sided of deletion in sperm manose receptor expression were identified. In motile sperm populations from fertile donors, a consistent two-stage pattern of manose receptor expression was observed: (i) the percentages of spermatozoa exhibiting head-directed binding of Man-FITC-BSA was dramatically increased between the time of isolation and the first 18 h of incubation in albumin-supplemented media (P < 0.0001) and (ii) further incubation did not, however, elicit an increased manose receptor expression above the levels observed at 18 h (P = 0.19). The first peak in this well defined pattern occurs in category I patients wherein the normal time course of manose receptor surface expression was delayed (fresh versus incubated 18 h, P = 0.99, not significant; fresh isolate or incubated 18 h versus incubated 3 days, P < 0.0001). The second defect identified resulted in abnormally low percentages of sperm expressing manose receptors on their heads at all incubation times examined (category 3a and b; fresh versus incubated 18 h, P = 0.18, not significant; fresh or incubated 18 h versus incubated 3 days, P = 0.66, not significant). In contrast, infertility category 2 men had defects in sperm function, in spite of the fact that the pattern of sperm surface manose receptor expression was indistinguishable from that of fertile donors (P = 0.31): the percentages of sperm binding Man-FITC-BSA over their head membranes increased from baseline to plateau values by 18 h of incubation (fresh versus incubated 18 h, P < 0.1); incubated 18 h versus incubated 5 days, P = 0.52). When histogram bars of the two categories were obtained from the representative individuals and analysed for the particular parameter in question.

acrosome reactions over that observed under standard incuba- tion conditions. These data indicate that sperm coating factors are not directly related to the observed IVF fertilization failures and do not appear to affect manose receptor expression of the sperm surface.

We postulated that prolonged incubation of patients’ spermatozoa in the presence of known steroid acceptors could affect a sufficient reduction in membrane cholesterol content so as to permit surface expression of manose receptors. Therefore, in a second series of experiments, conditions for prolonged incubation which promoted increased manose receptor expression and under which significant sperm motility was retained were determined using fertile donor spermatozoa (n = 21). Aliquots from motile sperm populations were incubated in Ham’s F-10 medium supplemented with 30 mg/ml or 5 mg/ml BSA for 1, 2 and 3 days at 37°C or at room temperature.

Figure 3. Spontaneous acrosome loss was measured in freshly isolated motile sperm populations (FI) and in duplicate aliquots incubated in Ham’s F-10 containing 30 mg/ml delipidated HSA (-INCR). Mixed aliquots from incubated spermatozoa were also assayed for their ability to undergo in acrosome reaction following exposure to 26.5 ±mM Man-FITC-BSA, 77 µM of manose monosaccharide (+MAN), to 1 μg/ml progesterone (+P), to 0.5% EHOB (+ETOH), and/or to 3 µM ionomycin (+IONOMYCIN). Where incubation times are not indicated, aliquots have been incubated for 18 h at 3°C. RMANOVA with subsequent pairwise comparisons conclusively demonstrates that significant differences exist for mean levels of spontaneous and induced acrosome loss between motile sperm populations from fertile donors and infertility patients (P < 0.0001). The level of spontaneous acrosome loss in specimens from fertile donors is significantly increased by incubating incubations (P < 0.0015) and the percentage of incubated incubations undergoing an acrosome reaction is further increased by application of any of the three agonists tested but not by cAMP solution (P < 0.0001). In contrast, in category I, sperm prolonged incubation is required to attain similar spermatozoa in the percentages of spermatozoa exhibiting spontaneous or induced acrosome reaction (+FI versus INCR 1 day, P = 0.09, not significant; FI or INCR 1 day versus INCR 3 days, P < 0.0001). In category 2 sperm, no differences in acrosome loss is observed between freshly isolated and incubated aliquots (spontaneous acrosome loss, FI versus INCR 1 day or 3 days, P = 0.3); significance; for the analysis versus incubated manose receptor treated, P = 0.71; not significant). In category 3 spermatozoa, neither averaged percentages of spontaneous acrosome reactions nor responsiveness to manose or progesterone treatment is observed following capacitating incubations. (P = 0.43, not significant). However, the response of some specimens to ionomycin will be indistinguishable from that of fertile donors (P = 0.07, not significant), resulting in false-positives, (e.g. category 3b). Histogram bars for SD are depicted in Figure 2.

At both incubation temperatures, 2d in both media, manose receptor expression increased from baseline to plateau values by day 1 of incubations (P < 0.0001), not shown). Those plateau values were unchanged at 2 and 3 days of incubation (P = 0.98, not significant). Further, no difference in the percentage of sperm binding Man-FITC-BSA on their head membranes was detected between the two incubation temperatures (P = 0.59, not significant). However, although incubation for 3 days at 37°C completely abolished sperm motility, at least 50% of room-temperature-incubated sperm remained motile. Optimum conditions for prolonged incubation were thus defined as 3 days at room temperature in media supplemented with 30 mg/ml of delipidated HSA.

Patient specimens (n = 11) were then incubated under these optimum conditions and the resultant levels of manose receptor expression, acrosome- loss and relative free cholesterol.
content were compared with those at 0 time and after standard capitating incubations (Figures 2–4, typical results). Relative membrane cholesterol content was decreased to <0.3 per cell after 3 days of incubation, a value previously equated with fecundity (Benoff et al., 1993c) and was associated with significantly increased surface expression of mannose receptors and spontaneous acrosome reactions. In addition, exposure of 3 day incubated spermatozoa to polyvalent mannose ligands + mannose monosaccharide stimulated acrosomal exocytosis. Thus, prolonged incubation is associated with both acquisition of zona binding potential and the ability to undergo an acrosome reaction induced by zona ligands. These data support the hypothesis that the infertility in this patient subgroup is the result of a plasma membrane fluidity defect.

To confirm these observations, in a third series of experiments, the plasma membranes of fertile donor spermatozoa (n = 14) were hyperfused with cholesterol by overnight incubation at room temperature in the presence of 1 mg/ml C-BSA (Figure 5, typical results). Spermatozoa stored in C-BSA displayed surface Man-FITC-BSA binding characteristic of untreated, fresh swim-up preparations. When washed free of the inhibiting cholesterol and then incubated under capitating conditions for 3 days at room temperature, relative membrane free cholesterol content was reduced to <0.3 per cell (not shown). The percentage of spermatozoa exhibiting head-directed Man-FITC-BSA binding was simultaneously increased, comparable to that of spermatozoa never exposed to cholesterol (Figure 5A). Furthermore, while C-BSA suppresses both spontaneous and induced acrosome loss, cholesterol, unloading by 3 day incubations is associated with increases in these parameters (Figure 5B). These results mimic those obtained when spermatozoa from our patients and demonstrate that high α1-vels of non-esterified cholesterol are responsible for adhesion of spermatozoa associated with vissel fertility potential.

**Category 2: Reduced ability to undergo an acrosome reaction initiated by zona ligands, a post-mannose receptor expression blockage**

In a second category of infertile men with normoospermic semen parameters, the time course of capitating-associated increased mannose receptor expression is not significantly different from that observed in specimens from fertile donors. This patient category was identified by use of failed fertilization in IVF which was unexpected based on our pretest results: i.e. none out of 12 metaphase II oocytes fertilized following a standard IVF insemination based on normal acrosome morphology (Benoff et al., 1995c) and the fact that 33.8% of incubated spermatozoa exhibited normal membrane binding characteristics. The data in Figure 2 illustrate the similarities between mannose receptor expression by fertile donor spermatozoa (n = 25) and spermatozoa from this second patient group (n = 7). In motile sperm populations from category 2 semen, mannose receptor expression increased from baseline to plateau values by 18 h incubation. These findings mimic those obtained from fertile donors. The final percentages of acrosome intact incubated sperm in these patient specimens expressing head-
The ability to undergo an acrosome reaction appeared to be limiting in these patients. Typical findings are presented in Figure 3. Motile spermatozoa from category 2 patients differed in acrosome status from those of fertile donors in two important ways: Firstly, the percentage of spermatozoa exhibiting spontaneous loss of acrosome content was not increased after overnight incubation in albumin-supplemented media; <5% of viable spermatozoa had undergone an acrosome reaction at 0 time or after 18 h. In contrast, fertile donors spermatozoa exhibit a significant rise in the percentage of spontaneous acrosomal reactions following overnight incubation. Secondly, and more importantly, incubated acrosome-intact motile spermatozoa expressing head-directed membrane receptors could not be induced to acrosome react by exposure to polyvalent mannoside ligands in the presence of free β-mannosidase, whereas in fertile donors, such treatment results in a significant increase in acrosome loss. Neither mannoside receptor aggregation and translocation to the equatorial-post-acrosomal segment of the sperm head (not shown; paired t-test, P = 0.51, not significant) nor loss of acrosome content was induced by these conditions which mimic sperm binding to the zona pellucida. Prolonged incubation also did not alter this refractory state. Moreover, these abnormal parameters persisted over time in cases where it was possible to obtain multiple specimens from the same patient (n = 4). These data provide further evidence that the physiological acrosome reaction occurs after zona pellucida binding and allow discrimination between a defect in capacitation potential (category 1) and an inability to undergo an acrosome reaction when mannoside ligand receptors are expressed on the sperm head (category 2).

The unifying feature of patients with category 2 sperm deficits is that, on clinical examination, they were found to have a varicocele (Class 1 varicocele; Benoff et al., 1995a). In the three cases where a category 2 patient elected to undergo a varicocelectomy repair, the underlying sperm defect in the ability to undergo a spontaneous or induced acrosome reaction persisted after surgery. In terms of mean values, only 2.30 ± 1.29% and 2.34 ± 1.5% of spermatozoa respectively, from matched pre-ligation and post-ligation specimens exhibited spontaneous loss of acrosome content. More importantly, only an additional 1.3 ± 1.1% of motile spermatozoa obtained pre-ligation and 0.7 ± 0.4% in matched post-ligation specimens responded to mannoside exposure by undergoing an acrosome reaction (paired t-test, P = 0.50, not significant).

A relationship exists between the surface distribution of sperm surface mannoside binding sites (Figure 1B) and the anti-myoosin antibody immunocytochemical staining patterns (Figure 6, middle panel) of unfixed, Triton-permeabilized human spermatozoa from fertile donors (n = 6), which profiled the tropism of anti-myoosin antibody reactivity also correlates with the state of the acrosome (Figure 6, lower panel). To determine whether the inability to undergo an acrosome reaction stimulated by mannoside treatment was the result of reduced expression of other integral membrane proteins with myosin-like motors which may associate with the mannoside receptor (Benoff et al., 1996a), we analysed the expression of this protein family in sperm from fertile donors and category 2 infertility patients by Western blot analysis. No difference in the distribution of integral membrane proteins with

![Figure 5](https://example.com/image.png)

**Figure 5.** The effects of high membrane cholesterol content on mannose receptor expression in spermatozoa from category 1 infertility patients (see Figures 2–4) can be mimicked in fertile donor spermatozoa by incubation in cholesterol-supplemented BSA (C-BSA) which raises 2- to 5-fold the non-esterified cholesterol content of the sperm plasma membrane (Benoff, 1993; Benoff et al., 1993a, 1994b, 1995b). (A) The percentage of spermatozoa exhibiting head-directed Man-FITC-BSA binding (patterns I + II combined) in control aliquots in fertile spermatozoa were compared with those of duplicate aliquots incubated for 7 days at room temperature in 1 mg/ml C-BSA prior to exposure to capacitating conditions. The percentages of sperm binding Man-FITC-BSA in control and treated aliquots were not significantly different either at 0 time or after capacitating incubations (MANOVA statistics; respectively, F = 0.1084 and P = 0.3049, not significant). In contrast, when control or C-BSA treated aliquots were subjected to capacitating incubations ("INCUBATED"), a significant increase was observed in the percentages of spermatozoa exhibiting head-directed Man-FITC-BSA binding (P < 0.0001). Importantly, the percentages of incubating spermatozoa expressing mannose receptors did not differ significantly between control and C-BSA treated aliquots (P = 0.3498, not significant). (B) High membrane cholesterol content inhibits acrosome exocytosis induced by exposure to polyvalent mannoside ligands + sialic acid binding. Pattern II Man-FITC-BSA cannot be converted to pattern III by the addition of 75 nM β-mannose. Capacitating incubations (3 days at room temperature) which reduce plasma membrane free cholesterol content to <0.001 mmol/100 mg spermatozoa (Benoff et al., 1993a) are associated with the acquisition of the ability to convert, in the presence of 75 nM β-mannose, pattern II to pattern III Man-FITC-BSA binding with undetectable loss of acrosome content. Located mannoside binding sites were also similar to the fertile controls. In all cases, >20% of acrosome-intact spermatozoa bound the mannosylated BSA probe.
S. Benoff et al.

Figure 6. Anti-myosin antibody binding to acrosome-intact and acrosome-reacted human spermatozoa by double labelling; labelled spermatozoa were viewed at ×600 and photographed at an original magnification of ×1500 on 400 ASA film with exposure times for fluorescein and rhodamine images respectively, of 36 and 4 s. (Upper panel) Phase-contrast image of unfixed, Trypan- 
pompeasublistened capacitated human spermatozoa from a fertile donor. (Middle panel) The same field viewed with UV-excited fluorescein 
aptice-reveals binding of anti-myosin antibodies occur on the 
basal sperm head in two patterns similar to those observed for 
head-directed Man-FITC-BSA binding (see Figure 1; Benoff et al., 1992a,b; paterm II [large arrow], uniform labelling covering the region of 
the acrosomal cap, and pattern III [small arrow], labelling in the 
equatorial segment only. (Lower panel) The corresponding FITC- 
BSA labelling patterns indicate that sperm binding anti-myosin 
accounts in patterns I and acrosome-intact [large arrow] whereas 
these exhibiting pattern III anti-myosin antibody binding are 
acrosome-reacted [small arrow]. In all donors examined, the same 
pattern of results was evident; 100% of sperm with pattern II anti- 
myosin reactivity were acrosome intact whereas 100% of 
sperrnatozoa exhibiting anti-myosyn antibody binding in pattern III 
were acrosome reacted. Fapa for each donor was 1.00, indicating 
complete agreement between the distribution of myosin epitopes 
and acrosomal status. Thus, as previously reported for the human 
sperm mannosor receptor (Benoff et al., 1995c), the topography 
of anti-myosin antibody-reactivity correlates with the state of the 
acrosome.

myosin motors was observed between control and category 2 
spermatozoa (not shown); paired vent, control versus patient 
spermatozoa, P = 0.94, not significant. Thus, the inability 
to undergo induced acrosome loss could not be attributed to 
alterations in gene expression of proteins integral to the sperm 
plasma membrane.

Category 3: Reduced expression of a family of integral sperm 
membrane proteins

Semen from patients in this last category of male factor 
infertility differs from that of the two other categories above 
in that the abnormalities in sperm function were observed in 
patients both with normal spermatogonem semen parameters (n = 
7; category 3a) and those with teratospermia without varicocele 
(n = 2; category 3b). This patient group is the most interesting 
by far. Category 3 patient spermatozoa differed from fertile 
donor spermatozoa in all parameters examined.

Mannose receptor expression by category 3 patient spermato-
zoa did not follow the time course documented for fertile donor 
population (Figure 2,typical results). Only low percentages of 
spermatozoa from motile populations expressed mannosese 
receptors on their head plasma membranes. Overnight incuba-
tion in albumin-supplemented media failed to elicit an increase in 
the percentage of spermatozoa capable of binding Man-
FITC-BSA (patient versus fertile donor spermatozoa, 
P < 0.0012). Prolonged incubation for 3 days also failed to 
increase the number of sperm with mannosese binding sites on 
their surface (patient versus control, P < 0.0012). As would be 
expected, at both time points, such specimens also failed to 
dergo an acrosome reaction stimulated by exposure to polyvalent 
mannose ligands + α-mannose monochoptotide (Figure 3, typical results). Thus, the 3 day incubation period 
readily permits discrimination between category 1 and category 
3 sperm function deficits.

In spermatozoa from fertile donors, mannosese receptors and 
nuclear-progestrone receptors exhibit the same topograph-
ical distributions on the sperm plasma head. More importantly, 
the mannosese receptor and the non-nuclear progestrone receptor 
are co-expressed and form a complex on the surface of the 
capacitated spermatozoa (Benoff et al., 1995c). To determine 
whether progestrone receptor expression was coordinate 
ally altered in category 3 spermatozoa, we examined the ability of 
progestrone to stimulate acrosomal exocytosis (n = 4). Results 
were compared with the level of spontaneous acrosome loss 
in unregulated and incubated aliquots and that observed following 
mannose treatment. To determine whether any reductions in 
inuced acrosome loss could potentially be attributed to 
alterations in calcium influx, duplicate aliquots were exposed to 
the calcium ionophore ionomycin. Findings from this 
analysis are summarized in Figure 3.

Mannose, progestrone and ionomycin, but not carrier solv-
eints, stimulated acrosomal exocytosis in incubated motile 
sperm populations from fertile donors. Exposure of capacitated 
category 3 spermatoza to progestrone, however, did not 
result in an increase in acrosome loss over that produced by 
capacitation incubations alone. In contrast, spermatozoa from 
some of these infertility patients exhibiting acrosomal 
exocytosis after ionomycin exposure. Thus use of ionomycin, 
but not mannosse or progesterone, would result in false- 
positive findings.

These data suggested that category 3 patient spermatozoa 
were coordinate depleted of receptor proteins capable of
binding mannose or progesterone. The possibility existed, however, that the inability of patient spermatozoa to bind Man-FITC-BSA was simply the result of the failure to externalize subplasmalemmal space following synthesis. Thus, the inability to undergo a progesterone-stimulated acrosome reaction would then be related to the failure to normally form mannose ligand/ progesterone receptor complexes on the sperm surface. To further explore these alternatives and to determine whether the accumulation of other as yet unidentified members of the sperm membrane myosin-like superfamily was coordinately affected, Western blots prepared with patient sperm proteins were probed with anti-myosin antibodies. The anti-myosin antibody-reactive molecular species present in total protein extracts in category 3 spermatozoa were compared with those of fertile donors (Figure 7, typical results). Irrespective of whether the protein extracts were derived from normozoospermic or teratozoospermic patient semen, the densitometric tracings of the stained blots demonstrated the specific reduction in all protein species reactive with anti-myosin antibodies in these nine patients. We calculated the 95% confidence interval for these nine observations, and found that 66–100% of all motile sperm populations failing to exhibit increased acrosomal exocytosis following exposure to mannose and progesterone ligands would also not express the remaining six to eight related membrane proteins. These data support the concept that the expression of unconventional myosin-like human sperm membrane proteins is coordinately regulated and that all members of this protein superfamily are absent in category 3 spermatozoa.

Discussion
Failed fertilization in couples with unexplained infertility is largely unexplored. We have employed such pathological semen as a model for the further understanding of sperm– egg interactions and have demonstrated that some cases of unexplained infertility truly represent male factor cases with normal sperm parameters, but with specific deficits in sperm function (Benoff et al., 1993a,c, 1994b, 1995b). Herein, we extend our findings and investigate retrospectively, by comparison with semen specimens that have fertilized oocytes in IVF, the mechanisms underlying complete IVF fertilization failure in 27 couples. Three assays were performed and a flow chart is provided to illustrate the indications for their use (Figure 8). These three assays differ from sperm function tests performed elsewhere (Liu and Baker, 1992; Schrader and Kesner, 1993) in that we employ molecular markers of sperm membrane function rather than indirect measures such as the zona-free hamster oocyte penetration test or the hypo-osmotic swelling test. In addition to the discovery of iogeographic infertility associated with therapeutic administration of calcium channel blockers (Benoff et al., 1994b, 1995b; Heschlag et al., 1995), use of these techniques has permitted the identification of three distinct categories of naturally-occurring human male factor infertility. A further difference between our methodology and that of others is that our results can predict fertilization outcomes in subsequent IVF cycles. Thus, the three categories of male infertility described herein allow us to assign males to one of two separate insemination strategies: (i) prolonged incubation prior to standard IVF, and (ii) directly to ICSI. The protocols we describe for assessment of sperm function are independent of sperm morphology. They measure both the potential of sperm populations to bind the human zona pellucida and to undergo acrosome reactions after zona binding. The acrosome reaction has been recognized as an essential feature of sperm– egg interactions in animal species. In man, where examination of the status of acrosome integrity requires special stains, electron microscopy has shown that only acrosome-reacted sperm fuse with zona-free hamster oocytes (Singer et al., 1985). Historically, ionophores, such as A23187, have been used to promote acrosome reactions as measured by human sperm fusion with zona-free hamster oocytes (Aitken et al., 1984). Ionophores, unfortunately, short circuit sperm capacitation events which physiologically precede acrosomal exocytosis: ionophores induce acrosome reactions in freshly isolated uncapacitated and in capacitated sperm to equal degrees (Bielfeld et al., 1994). Ionophore assays thus cannot identify those men whose sperm would not undergo a physiological acrosome reaction (e.g. category 3). Therefore, we
believe that isoantigens are of limited value for male fertility
evaluation. In contrast, micromolar quantities of polyvalent
mannose-lectins and rabbitmolar amounts of mannose monosac-
charide used together to mimic the effects of solubilized
zona pellucida glycoproteins upon capacitated, but not freshly
isolated, spermatozoa from fertile men, with similar rates of
exocytosis induction (minutes up to 2 h) and similar final
percentages of acrosomes induced to react (Benoff et al.,
1993c). The latter assay has aided in the identification of three
subgroups of male factor patients falling under the rubric of
‘unexplained infertility’.

The first patient category is defined by a delayed time course
of mannosel lectin surface expression. This is associated with
extremely high membrane cholesterol content, so that days
rather than hours of capacitating incubations are required to
produce cholesterol-depleted membranes with fusogenic
potential (Benoff, 1993; Benoff & Benoff, 1993a, 1995b; Herschlag
et al., 1995). As illustrated in Figures 3 and 4, such prolonged
incubation is associated with increased mannosel lectin expres-
sion on the sperm head surface and the acquisition of the
ability to undergo a mannosel-initiated acrosome reaction. With
regard to category 1 treatment modality, in a test case examined
an IVF cycle subsequent to a previous IVF cycle with
fertilization failure, four out of four oocytes fertilized when
incubated with the husband’s spermatozoa which were preincubated for three days at room temperature prior to egg
sperm. Likewise, the importance of tests for sperm function
and incubation strategies on IVF outcome is illustrated by a
couple referred to us after four cycles of failed fertilization in
vitro using standard insemination protocols, including a
failed tubal insertion (SUZI) cycle and an ICSI cycle with
poor ‘fertilization’. In each prior cycle, 20–30 oocytes were
recovered. Conventional semen parameters showed a normal-
zooospermic male with excellent swim-up populations of at
least 30% normal oval spermatozoa and few tapering head
shapes. Our tests of sperm function, however, showed only low
levels of mannosel binding and substantially acrosome
reactions after 18 h of incubation. Classified as a possible
category 1 male, the husband’s spermatozoa were processed
by swim-up and incubated for 30 h at 37°C in 15% serumF-10
medium prior to in-vitro insemination at 10°egg; all five ova
fertilized normally and subsequently cleaved. The husband’s
spermatozoa prepared on the day of egg retrieval were inseminated by ICSI; two out of seven eggs fertilized.

Zoos-thawed donor spermatozoa fertilized 10 out of 10
ova. Comparable 100% fertilization with 30 h preincub-
ated/husband’s spermatozoa and 5 h-incubated donor spermato-
zoa indicate that the husband’s spermatozoa behave like those of
category 1 males whose previous IVF failures are related to
the cholest erol content of their spermatozoa.

Our data thus help to explain prior observations of (i) the
association between infertility and high cholesterol content of
human semen (Hosoda et al., 1981; Padton et al., 1989); and
(ii) the manner sperm end of spermatozoa containing positive”
SPA tests into “positive” ones (Bolanos et al., 1983). Johnson
et al., 1984). We conclude that category 1 men may benefit
from preincubation and high dosage compensation to achieve
normal fertilization rates, eliminating the need for micro-
manipulation inseminations.

In category 2, the percentages of motile incubated spermato-
zoa expressing mannosel binding sites on their heads are
equivalent to that observed for fertile donors. However, in
contast to the latter, category 2 spermatozoa do not undergo an acrosome reaction stimulated by polyvalent mannosel ligands
and a mannose monosaccharide. Spermatozoa from this patient
category can bind to the zona pellucida but apparently do not
undergo an acrosome reaction following zona binding nor
exhibit zona pellucida penetration. Men with such defective

1914
zona entry characteristics have been previously described (e.g., Overstreet et al., 1980; Frankenhaeuser et al., 1991; Liu and Baker, 1994b; Liu et al., 1995) and in contrast to our category 2, in these reports: (i) the rates of spontaneous acrosome loss in freely-swimming spermatozoa in patient and control specimens were similar; and (ii) it was suggested that the blockage in induced acrosome loss occurred subsequent to the major calcium ion transient (Thomson and Meizel, 1988) required for acrosome reaction (Liu and Baker, 1994b). We believe that our category 2 patients represent a crisis of sperm function deficits which differ from those reported above because the levels of spontaneous acrosome loss in our cases are considerably lower than that observed in fertile control specimens. We believe, therefore, that the deficit described as category 2 occurs prior to calcium influx into the spermatozoa. As polyvalent ligands are unable to effectively crosslink membrane receptors and induce aggregation, and because, importantly, there was no associated loss of acrosome content in the small percentage of spermatozoa in which membrane receptors had migrated to the equatorial segment (Bennett et al., 1995e). We have seen identical abnormalities in acrosome loss in cases of iatrogenic infertility associated with therapeutic administration of calcium ion channel blockers, e.g. nelfipine (Bennett et al., 1994b; 1995c; Herschlag et al., 1985), where we attributed the isolated percentages of acrosome loss following physiological ligand exposure to a disruption of the signal transduction pathway.

We find it striking that to date, all patients seen in category 2 have had varicoceles. An association between acrosome reaction insufficiency and varicocele has been previously noted (Rodgers et al., 1985; Vigil et al., 1994). Semen quality in varicocele patients is often characterized by increased percentages of spermatozoa with tapering head forms, which are a predictor of the deleterious effect of varicocele (Nathalir et al., 1991). However, the presence of varicocele is not always associated with infertility as the same semen abnormalities can be observed in both fertile and infertile men with varicocele (Nagao et al., 1986). More importantly, some infertile men with varicocele respond to varicocele correction with an improvement in semen quality and consequent fertility (Pryor and Howard, 1987; Geddie et al., 1992; Marlow et al., 1992), while others remain infertile (Baker et al., 1985). These observations suggest that the infertility of some men which is currently associated with the presence of a varicocele may not solely be due to their varicocele but rather to an existing sperm defect that is amplified by the pathological condition (Gentile and Cockett, 1991). Although future studies performed over a long interval will be required to evaluate whether treatment of varicocele of category 2 patients conclusively fails to reverse the observed acrosome reaction insufficiency, three semen characteristics of category 2 patients strongly support this hypothesis. Firstly, in whole semen and in motile sperm populations isolated from category 2 patients, <15% tapering head forms are present. Secondly, the functional deficits described herein were specifically observed with motile spermatozoa with normal oval head forms. Thirdly, the acrosome reaction insufficiency of normal sperm from category 2 patients persisted after varicocele ligation. As the full complement of membrane proteins which express myosin-like epitopes was present in both fresh and incubated motile spermatozoa (Bennett et al., 1995a), we suggest that the hypothesized deficit in category 2 patients resides in the mechanisms regulating signal transduction or calcium ion influx. That co-factors actually contribute to the infertility associated with varicocele is demonstrated by our recent findings suggesting that a candidate gene may be responsible for a defect in trace metal regulation as the concentrations of seminal calcium are elevated and those of zinc decreased in infertile men with varicocele (Bennett et al., 1996). As quantitative molecular fingerprints in expression of integral sperm plasma membrane proteins occur among infertile men with varicocele (Bennett et al., 1995a, 1996), it is conceivable that additional co-factors will be identified.

Recent evidence indicates that the acrosome reaction is unlikely to be important for fertilization by ICSI (Ng et al., 1993; Liu et al., 1995). Our data support this conclusion. Successful fertilization with category 2 husband’s motile spermatozoa was obtained by rescue ICSI with four out of 13 oocytes being fertilized following failure to fertilize by standard insemination. Screening by the methodology described herein prior to the commencement of an IVE cycle will direct assignment of such patients to ICSI and markedly improve chances for fertilization over that obtained with standard IVF fertilization. In all cases from category 3, IVF fertilization was not obtained with standard insemination protocols. Our experience with alternate strategies with category 3 spermatozoa is limited. One couple each from category 3a and 3b have returned for a total of three IVE cycles. For the couple from category 3a with multiple failed IVF attempts elsewhere, two out of three oocytes fertilized with donor spermatozoa whereas only one out of five oocytes fertilized with the husband’s spermatozoa following direct ICSI. The category 3b trade had >79% tapering head forms. In the first repeat IVE cycle, >95 of the three oocytes were fertilized with the husband’s spermatozoa following standard IVF insemination with 60% 000 spermatozoa/oocyte and no fertilization was obtained by rescue ICSI. In a second cycle with category 3b semen, three out of nine oocytes were fertilized by direct ICSI while none out of four oocytes fertilized following standard IVF insemination with 1.5 x 106 husband’s spermatozoa/oocyte and only one embryo was obtained by rescue ICSI. Thus, although as in category 2, direct ICSI can improve the chances of conception, the rates are reduced. Whether donor trick-up could be considered, however, requires further investigation.

In category 3 infertile men, defective sperm function is associated with a failure to express a superfamily of 8–10 estrogen membrane proteins whose cytoplasmic domains all contain myosin-like motor sequences, i.e. actin binding and ATP nucleotide binding/ATPase activity tese (Bennett et al., 1994a) coupled with an abnormal cytoskeletal organization of the sperm head. By analogy with other cell types, these findings have important implications for the regulation of human sperm acrosome exocytosis in response to physiological agonists. Recent investigations of the cytoskeleton indicate that, in addition to serving a structural role, the membrane protein/cytoskeleton associations may regulate the activity of a number
of ion channels, either through direct interactions (Lacoste et al., 1995; Schweinert et al., 1994) or by interaction with components of second messenger systems (e.g. of Mander 1994). Membrane protein/cytoskeletal associations and second messenger systems also play a role in cell adhesion. An example of this can be found in the desmosomal cadherins, transmembrane glycoproteins involved in adhesion molecules containing binding sites that interact with a submembrane core protein (Goodwin et al., 1990). This interaction activates the tethering and confluence of these low affinity receptor proteins by the cytoskeleton into a high affinity junction complex (Goodwin et al., 1990; Maithe et al., 1994). The human sperm mannose receptor also possesses amino acid sequences that permit its interaction with an underlying cytoskeleton (Benoff et al. 1994b). Ligand-stimulated mannose receptor aggregation is normally associated with acrosome loss by human spermatozoa (Benoff et al. 1995a), and receptor aggregation activates secondary messenger systems that phosphorylate sperm proteins other than the mannose receptor (Benoff et al., 1995e). We propose that the interaction of the human sperm mannose receptor with an actin/intermediate filament functions both to regulate tight binding of human sperm to the zona pellucida and also to regulate calcium ion influx via signal transduction through the cytoskeleton.

The proposal (Zanen et al., 1991) that the sperm cytoskeleton serves to separate and prevent fusion of the outer acrosomal and plasma membranes is relevant, in light of findings in other cell types where the cytoskeleton regulates the docking and fusion of secretory granules with the plasma membrane (e.g. Baudin et al., 1975; Fath and Burgess, 1993; Sollner et al., 1993; Mualem et al., 1995). Cytoskeletal elements apparently participate in antigen redistribution associated with the acrosome reaction (Peedher et al., 1986; Ochs et al., 1986) and in experimental models for environmental associated infertility (Benoff et al., 1995d, 1996; S.Benoff and M.Baucia, unpublished observations), specific transition metal ions which inhibit accumulation of sperm proteins reactive with anti-myosin and anti-actin antibodies by fertile donor spermatozoa also suppress both spontaneous and induced acrosome loss. We suggest that the expression of the anti-myosin antibody reactive superfamily of 8-10 potentially core transmembrane proteins which bind to the sperm cytoskeleton would be in absolute requirement for acrosome exocytosis, and elucidate how the observed depletion in category 3 sperrnaatozae of this superfamily would be associated with a marked reduction in the ability of sperm to exhibit spontaneous or induced loss of acrosome content. We support Siller's (1995) advocacy of continued research, into the molecular and genetic basis underlying defective sperm function, with the aim of devising alternative treatment strategies for some male factor patients. We recognize that only a small number of patients have been studied here and that these data should be confirmed by randomized and prospective studies with higher numbers of patients (such a study is presently in progress). Nevertheless, we believe that a general application of our findings will help IVE with embyro transfer to maintain its proper place in the treatment of male factor infertility, and reduce the number of cases requiring micromanipulation or the use of donor spermatozoa.

Acknowledgements
S.B. was supported in part by National Institutes of Health Grant No. ES06100. Grateful appreciation is expressed to David L.Rosenfield, Gerald M.Schultz, Frances H.Toney, and Eliah Mardon for the contribution of clinical IVF results, to Marlin Lesser for additional statistical input, and to James W.Goniewicz and Leslie A.Goodwin for critical reading of the manuscript. We also acknowledge Pardeep Guthrie, Terri Palen, Georgiina Krapil, Bush Chespulis and Deborah Lloha for their technical assistance.

References
Received on September 28, 1995; accepted on April 4, 1996