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

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Polyvalent mannose ligands in the presence of free mannose act as zona pellucida agonists which rapidly induce acrosome exocytosis in competent motile human sperm from fertile donors following in-vitro capacitation. Quantification of the binding patterns of fluorescein isothiocyanate-labelled mannosylated albumins and of specific antisera which recognize mannose receptors and other related integral 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 sterol acceptors) confer the ability to fertilize. Cholesterol loading 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 acrosome reactions in response to mannose treatment. Interestingly, all category 2 males identified in this study have clinical varicocele. 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 superfamily of integral membrane proteins whose cytoplasmic tails have myosin motors as identified by amino acid sequence analysis and anti-myosin antibody reactivity. Their spermatozoa do not express mannose ligand receptors or undergo induced acrosome reactions. Fertilization with category 2 and 3 semen is only achieved by micromanipulation 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, occupation) 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 (Brugo-Olmedo *et al.*, 1990; Mattison *et al.*, 1990; Schrader and Kesner, 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 spermatozoa penetrate through the human zona pellucida (Liu and Baker, 1993). Both the cumulus oophorus and the zona pellucida play a role in the selection of those sperm with normal oval head forms which may ultimately undergo an acrosome reaction at the zona surface (Carrell *et al.*, 1993; Liu and Baker, 1994a). As a result, we (Benoff *et al.*, 1995e) and others (Kruger *et al.*, 1986, 1988; Oehninger *et al.*, 1988; Fiorentino *et al.*, 1994; Ombelet *et al.*, 1994; Ozgur *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 where defects in acrosome morphology can be detected by inspection, e.g. when >50% of spermatozoa had small acrosomes, it is still possible

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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 (Benoff *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 pellucida binding potential of motile sperm populations using a neoglycoprotein ligand, fluorescein (FITC)-conjugated mannosylated 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 (Benoff *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 capacitating incubation at 37°C and/or after 2–3 days incubation at room temperature.

Only a subpopulation of motile human spermatozoa are ever able to undergo an acrosome reaction (Benoff *et al.*, 1995c,e). Motile spermatozoa which can be induced to acrosome react in response to appropriate stimuli represent that 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 + D-mannose monosaccharide (Benoff *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 (Benoff *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 (Benoff *et al.*, 1994a). The third assay is also based on data, appearing herein, that the topographical 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/spectrin 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 sperm populations in fertile and infertile men by Western blot analysis of whole sperm protein extracts.

Three categories of abnormalities in mannose 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 insemination strategies could be devised based on our classification of sperm plasma membrane deficits.

Materials and methods

Media and chemicals

Modified Ham's F-10 medium (Formula No. 90-8050PG) 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, 1992) criteria.

Portions of semen specimens produced for diagnostic purposes were obtained from patients from two university hospital-based IVF-assisted reproductive technology programmes and from a male urology private practice. Spermatozoa positive for immunoglobulin (Ig)A and IgG anti-sperm antibodies (ASAs), detected by direct immunobead 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 (Benoff *et al.*, 1993a). 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 unremarkable; 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 morphology criteria advocated for IVF (Kruger *et al.*, 1986, 1988; Grow *et al.*, 1994; Ombelet *et al.*, 1994) as >14% of their spermatozoa had normal morphology. Informed consent was not required for these unidentified specimens.

Semen preparation for experimental studies

Fresh semen specimens, collected by masturbation after 2–3 days of abstinence, were subjected to routine semen analysis (WHO, 1992). Spermatozoa from fertile donors and from some of the urological practice patients were then selected for motility by a 'swim-up' method as described previously (Bronson *et al.*, 1982). The remainder of the motile populations analysed were obtained from semen frozen in *N*-tris(hydroxymethyl)methyl-2-aminoethane-sulphonic acid/tris (hydroxymethyl)amino-ethane (TEST)-yolk-buffered medium containing glycerol (Irvine Scientific, Santa Ana, CA, USA). In these cases, three step Percoll gradients, 40, 70, and 90% (0.7:0.7:1.5 m each) were overlaid with thawed semen diluted with Ham's F-10

(1:1, v/v) and centrifuged for 55 min at 300 *g*. Prior comparison of matched freshly ejaculated and frozen/thawed semen from fertile donors and from infertility patients has demonstrated that mannose binding, acrosome status and membrane cholesterol characteristics are unaffected by liquid nitrogen storage (Benoff *et al.*, 1993c).

Untreated ('fresh' or uncapacitated) 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-delipidated (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 centrifugation and their motility was assessed by phase-contrast microscopy.

Visualization of D-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. A7790) 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 at ×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 spermatozoa were ethanol-permeabilized and differentiated by subsequent reaction with 100 µg/ml rhodamine-labelled *Pisum sativum* agglutinin (RITC-PSA; Vector Laboratories Inc., Burlingame, CA, USA) in distilled 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 potential zona ligands on acrosome status, 75 mM D-mannose monosaccharide was included in the calcium-supplemented buffer as described above during pre-wash and Man-FITC-BSA surface labelling reactions (Benoff *et al.*, 1995e); (ii) to examine the effect of cumulus-secreted progesterone on acrosome status, spermatozoa were incubated for 20 min at 37°C in calcium-supplemented core buffer containing 1 µg/ml progesterone (Sigma No. P0130); control reactions were performed in the absence of progesterone as well as in the presence of 0.1% ethanol (the solvent used to prepare the initial concentrated progesterone stock solution) (Benoff *et al.*, 1993e); (iii) to examine the effect of a non-physiological stimulus on acrosome status, spermatozoa were exposed

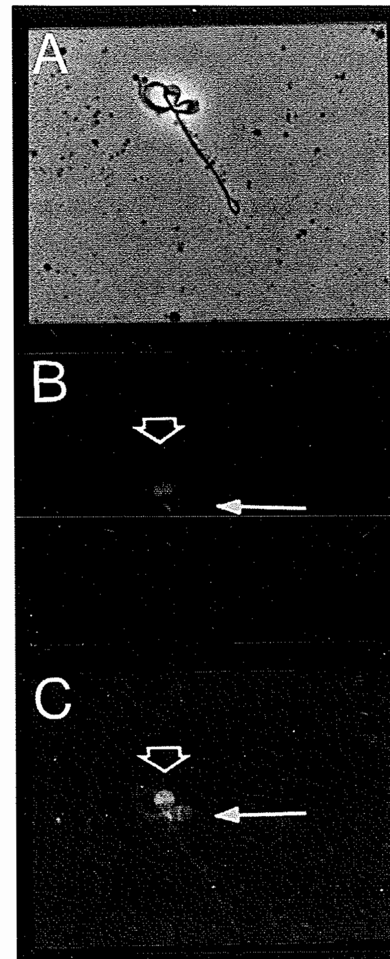

Figure 1. Photomicrographs taken with phase-contrast and with two UV-epifluorescence illuminations of capacitated human spermatozoa double-labelled with Man-fluorescein isothiocyanate (FITC)-bovine serum albumin (BSA) and rhodamine-labelled *Pisum sativum* agglutinin (RITC-PSA). (**A**) Phase-contrast 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 spermatozoon labelled in pattern III. (**C**) The corresponding RITC-PSA labelling patterns demonstrate that the spermatozoon 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).

to 3 µM ionomycin following the protocol of Thomas and Meizel (1988). Control aliquots were exposed to 0.5% ethanol, the solvent used to prepare the ionomycin stock solution.

Alternative conditions for capacitating incubations

Three procedures were used to strip the spermatozoa of any surface-bound inhibitory coating proteins, 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 (ionic strength = 390 mOsM) (Oliphant, 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 TEST-yolk buffer and incubated for 16–20 h at 4°C (Bolanos *et al.*, 1983; Johnson *et al.*, 1983; Carrell *et al.*, 1992).

In order to increase the proportion of spermatozoa undergoing the acrosome reaction, motile spermatozoa were incubated in Ham's F-

10 containing 15% human follicular fluid for 16–20 h at 37°C in 5% CO_2/95% air (Blumenfeld and Nahhas, 1989).

Determination of sperm cholesterol concentration

Relative concentrations of sperm plasma membrane-associated, non-esterified cholesterol were determined by gas–liquid chromatography on an Rt_x-50 column (Restek Corp., Bellefonte, PA, USA) as previously described (Benoff *et al.*, 1993d). Prior observations indicate that neither the total sperm sterol content nor the increment 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 18–20 h in Ham's F-10 containing 1 mg/ml cholesterol-saturated BSA (C-BSA) (Benoff *et al.*, 1993a). 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 delipidated HSA as described above.

Indirect immunofluorescence cytochemistry

Spermatozoa were washed three times with PBS and smeared onto the well of a precleaned heavy Teflon-coated (HTC) microscope slide (#10-1179; Cel-Line Associates, Inc., Newfield, NJ, USA), and allowed to air dry. The buffer system employed for immunohistochemical labelling was adapted from Danto 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-7648) served as primary antibody; preimmune rabbit serum was employed as control. Spermatozoa were reacted sequentially with primary antibody and human serum protein-preadsorbed, 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-cytoskeletal protein antibodies with acrosomal status, spermatozoa were double-labelled with RITC-PSA (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 T_{MAX} 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 capacitation 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 results). Abnormally high concentrations of non-esterified cholesterol 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 0.14–0.74 per cell in untreated specimens and decreases to 0.11–0.35 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 reported to increase the penetration of zona-free hamster oocytes by human spermatozoa were examined for their ability to promote acrosome loss and also to increase surface expression of mannose receptors in comparison with standard swim-up and incubation in Ham's F-10 + 30 mg/ml HSA overnight at 37°C in 5% CO_2/95% 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

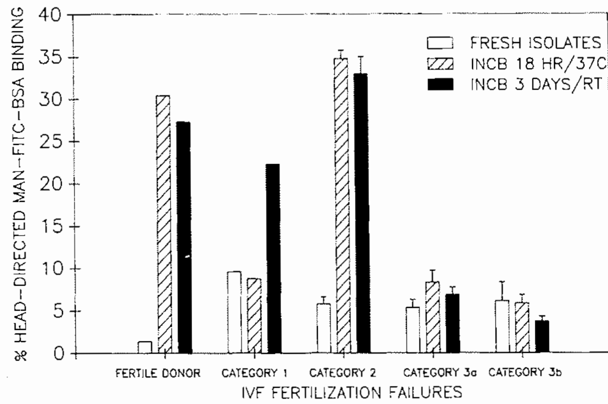

Figure 2. A repeated measures analysis of variance (RMANOVA) demonstrated that significant differences exist in mannose receptor expression between fertile donors and males who failed to fertilize oocytes in IVF ($P < 0.0001$). These differences were further analysed by pairwise contrast (paired t-test) and a minimum of two types of deficits in sperm mannose receptor expression were identified. In motile sperm populations from fertile donors, a consistent two-stage pattern of mannose 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 mannose receptor expression above the levels observed at 18 h ($P = 0.19$). The first deficit in this well defined pattern occurs in category 1 patients wherein the normal time course of mannose 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, $P < 0.0007$). The second deficit identified resulted in abnormally low percentages of sperm expressing mannose 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, infertile category 2 men had defects in sperm function, in spite of the fact that the pattern of sperm surface mannose receptor expression was indistinguishable from that of fertile donors ($P = 0.51$): 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, $P < 0.11$; incubated 18 h versus incubated 3 days, $P = 0.52$). When histogram bars have SD this indicates that $\geqslant 3$ specimens were obtained from the representative individual and analysed for the particular parameter in question.

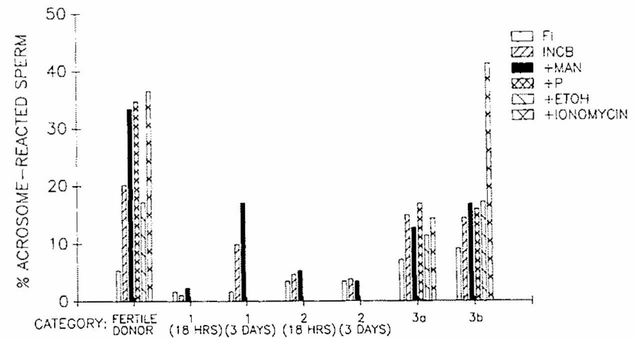

Figure 3. Spontaneous acrosome loss was measured in freshly isolated motile sperm populations ('FI') and in duplicate aliquots incubated in Ham's F-10 containing 30 mg/ml delipidated HSA ('INCB'). Matched aliquots from incubated spermatozoa were also assayed for their ability to undergo an acrosome reaction following exposure to 26.5 μM Man-BSA + 75 mM D-mannose monosaccharide ('+MAN'), to 1 μg/ml progesterone ('+P'), to 0.5% EtOH (+'ETOH'), and/or to 3 μM ionomycin (+IONOMYCIN). Where incubation times are not indicated, aliquots have been incubated for 18 h at 37°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 capacitating incubations ($P < 0.0015$) and the percentage of incubated spermatozoa undergoing an acrosome reaction is further increased by application of any of the three agonists tested but not by carrier solvent ($P < 0.0001$). In contrast, in category 1, sperm prolonged incubation is required to attain similar increases in the percentages of spermatozoa exhibiting spontaneous or induced acrosome reactions (FI versus INCB 1 day, $P = 0.99$, not significant; FI or INCB 1 day versus INCB 3 days, $P < 0.0007$). In category 2 spermatozoa, no difference in acrosome loss is observed between freshly isolated and incubated aliquots (spontaneous acrosome loss, FI versus INCB 1 day or 3 days, $P = 0.83$, not significant; incubated control versus incubated/mannose treated, $P = 0.71$, not significant). In category 3 spermatozoa, neither increased percentages of spontaneous acrosome reactions nor responsiveness to mannose 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 as described in Figure 2.

acrosome reactions over that observed under standard incubation conditions. These data indicate that sperm coating factors are not directly related to the observed IVF fertilization failures and do not appear to mask mannose receptors on the sperm surface.

We postulated that prolonged incubation of patients' spermatozoa in the presence of known sterol acceptors could effect a sufficient reduction in membrane cholesterol content so as to permit surface expression of mannose receptors. Therefore, in a second series of experiments, conditions for prolonged incubation which promoted increased mannose receptor expression and under which significant sperm motility is 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. At both incubation temperatures, and in both media, mannose receptor expression increased from baseline to plateau values by day 1 of incubation ($P < 0.0001$; not shown). These 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 mannose receptor expression, acrosome loss and relative free cholesterol

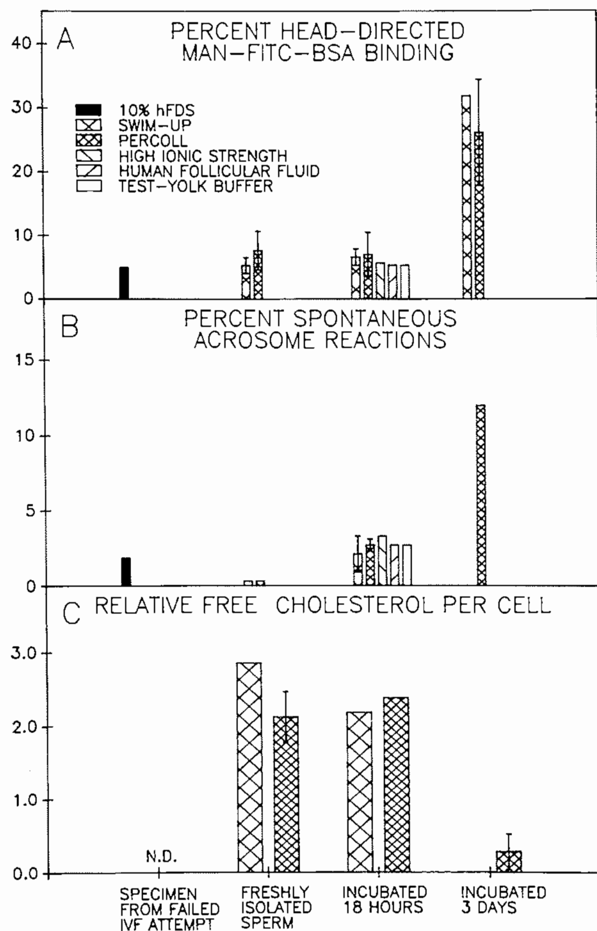

Figure 4. Repeated analysis of motile sperm populations from category 1 normozoospermic in-vitro fertilization (IVF) failures. Effect of treatment/incubation conditions on (**A**) mannose-ligand receptor expression, (**B**) acrosome status and (**C**) the relative free cholesterol content of the sperm plasma membrane. Complete experimental details are given in the Materials and methods section. (**A**) Spermatozoa incubated under standard laboratory capacitating conditions (18 h at 37°C in 5% CO_2 in air in Ham's F-10 + 30 mg/ml HSA) did not exhibit an increase in Man-FITC-BSA surface labelling (mean 0 time versus mean 18 h, 7.4 + 3.2% versus 7.4 + 2.6%; RMANOVA statistics, $P = 0.9898$, not significant). Alternate treatment/incubation conditions had no effect on the percentage of spermatozoa exhibiting Man-FITC-BSA binding (mean = 7.0 + 2.7%; RMANOVA statistics: standard incubation versus various treatments, $P = 0.6264$, not significant). In contrast, after 3 days incubation at room temperature the percentage of spermatozoa surface-labelled with Man-FITC-neoglycoprotein is markedly increased (mean 27.3 + 6.5%; RMANOVA statistics: 0 time versus 3 days, $P < 0.0007$). (**B**) Spermatozoa incubated under standard laboratory capacitating conditions did not exhibit an increase in the level of spontaneous acrosomal exocytosis (mean 0 time versus mean 18 h, 1.27 + 1.3% versus 2.1 + 1.2%; RMANOVA statistics, $P = 0.9868$, not significant). Alternate treatment/incubation conditions also failed to increase the percentage of spermatozoa exhibiting spontaneous acrosome reactions (mean = 2.7 + 2.7%; RMANOVA statistics: standard incubation versus various treatments, $P = 0.5926$, not significant) whereas 3 days incubation at room temperature was associated with a dramatic increase in spontaneous acrosomal exocytosis ($P < 0.0007$). (**C**) Abnormally high concentrations of free cholesterol are detected both before and after incubation under standard laboratory capacitating conditions (2.4 ± 0.5 versus 2.3 ± 0.1 per cell respectively) whereas after 3 days incubation at room temperature free cholesterol was reduced to <0.3 per cell ($P < 0.005$). Histogram bars for SD as described for Figure 2.

content were compared with those at 0 time and after standard capacitating 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 hyperloaded 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 capacitating 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). Further, 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 with spermatozoa from our patients and demonstrate that high levels of non-esterifed cholesterol are responsible for inhibition of expression of sperm characteristics associated with 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 normozoospermic semen parameters, the time course of capacitation-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.*, 1995e) and the fact that 33.8% of incubated spermatozoa exhibited normal mannose 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-

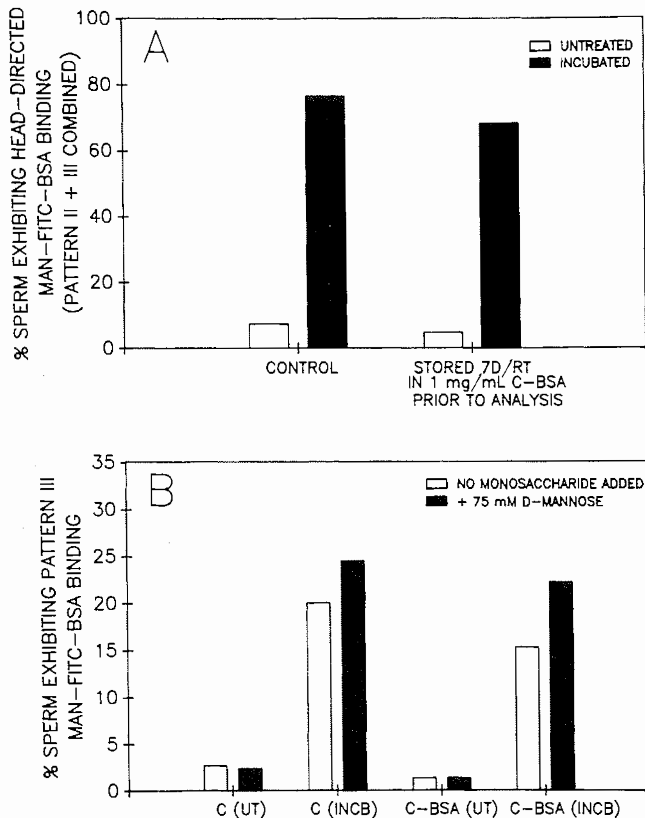

Figure 5. The effects of high membrane cholesterol content on mannose receptor expression in spermatozoa from category 1 infertility patients (see Figures 2–4) can be mimicked in fertile donor spermatozoa by incubation in cholesterol-saturated BSA (C-BSA) which raises 2- to 5-fold the non-esterified 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 II + III 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 (RMANOVA statistics: respectively, $P = 0.1084$ and $P = 0.9498$, 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 ($P = 0.9498$, not significant). (**B**) High membrane cholesterol content inhibits acrosome exocytosis induced by exposure to polyvalent mannose ligands + mannose monosaccharide: pattern II Man-FITC-BSA cannot be converted to pattern III by the addition of 75 mM D-mannose. Capacitating incubations (3 days at room temperature) which reduce plasma membrane free cholesterol content to <0.001 μmol/10^9 spermatozoa (Benoff *et al.*, 1993a) are associated with the acquisition of the ability to convert, in the presence of 75 mM D-mannose, pattern II to pattern III Man-FITC-BSA binding with simultaneous loss of acrosome content.

located mannose binding sites was also similar to the fertile controls. In all cases, >20% of acrosome-intact spermatozoa bound the mannosylated BSA probe.

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 donor spermatozoa exhibit a significant rise in the percentage of spontaneous acrosome reactions following overnight incubation. Secondly, and more importantly, incubated acrosome-intact motile spermatozoa expressing head-directed mannose receptors could not be induced to acrosome react by exposure to polyvalent mannose ligands in the presence of free D-mannose monosaccharide, whereas in fertile donors, such treatment results in a significant increase in acrosome loss. Neither mannose 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 refractive 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 mannose 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 I varicocele; Benoff *et al.*, 1995a). In the three cases where a category 2 patient elected to undergo a varicocele 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 ± 1.2% 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 mannose exposure by undergoing an acrosome reaction (paired *t*-test, $P = 0.50$, not significant).

A relationship exists between the surface distribution of sperm surface mannose binding sites (Figure 1B) and the anti-myosin antibody immunocytochemical staining patterns (Figure 6, middle panel) of unfixed, Triton-permeabilized human spermatozoa from fertile donors ($n = 6$). In addition, the topology of anti-myosin 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 mannose treatment was the result of reduced expression of other integral membrane proteins with myosin-like motors which may associate with the mannose receptor (Benoff *et al.*, 1994a), 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

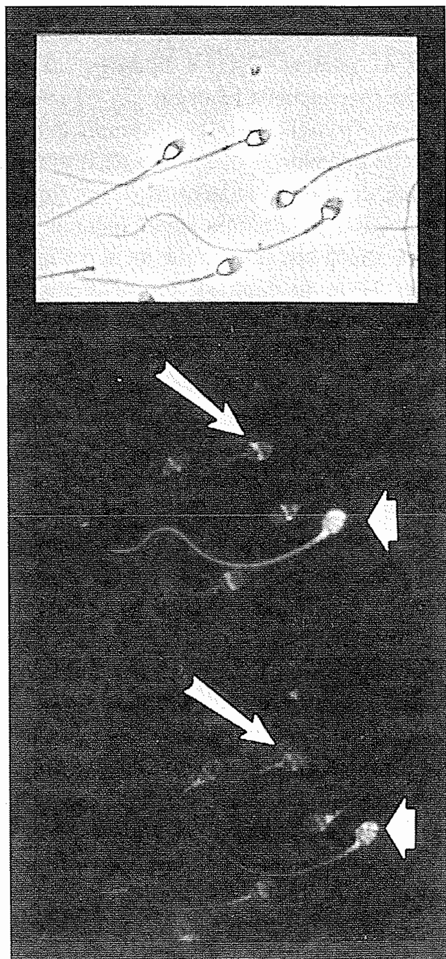

Figure 6. Anti-myosin antibody binding on acrosome-intact and acrosome-reacted human spermatozoa by double labelling: labelled spermatozoa were viewed at ×600 and photographed at an original magnification of ×1500 on 400 ASA film with exposure times for fluorescein and rhodamine images respectively, of 36 and 4 s. (Upper panel) Phase-contrast image of unfixed, Triton-permeabilized capacitated human spermatozoa from a fertile donor. (Middle panel) The same field viewed with UV-epifluorescence optics reveals binding of anti-myosin antibodies occurs on the human sperm head in two patterns similar to those observed for head-directed Man-FITC-BSA binding (see Figure 1; Benoff *et al.*, 1993b,d): pattern II (large arrow), uniform labelling over the region of the acrosome cap; and pattern III (small arrow), labelling in the equatorial segment only. (Lower panel) The corresponding RITC-PSA labelling patterns indicate that sperm binding anti-myosin antibodies in pattern II are acrosome-intact (large arrow) whereas those 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 spermatozoa exhibiting anti-myosin antibody binding in pattern III were acrosome reacted. Kappa 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 mannose receptor (Benoff *et al.*, 1995e), the topology 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 *t*-test, 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 normozoospermic 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 spermatozoa did not follow the time course documented for fertile donor populations (Figure 2, typical results). Only low percentages of spermatozoa from motile populations expressed mannose receptors on their head plasma membranes. Overnight incubation 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 mannose binding sites on their surface (patient versus control, $P < 0.0012$). As would be expected, at both time points, such specimens also failed to undergo an acrosome reaction stimulated by exposure to polyvalent mannose ligands + D-mannose monosaccharide (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, mannose receptors and non-nuclear progesterone receptors exhibit the same topographical distributions on the human sperm head. More importantly, the mannose receptor and the non-nuclear progesterone receptor are co-expressed and form a complex on the surface of capacitated spermatozoa (Benoff *et al.*, 1995c). To determine whether progesterone receptor expression was coordinately altered in category 3 spermatozoa, we examined the ability of progesterone to stimulate acrosomal exocytosis ($n = 4$). Results were compared with the level of spontaneous acrosome loss in untreated and incubated aliquots and that observed following mannose treatment. To determine whether any reductions in induced 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, progesterone and ionomycin, but not carrier solvents, stimulated acrosomal exocytosis in incubated motile sperm populations from fertile donors. Exposure of capacitated category 3 spermatozoa to progesterone, however, did not result in an increase in acrosome loss over that produced by capacitating incubations alone. In contrast, spermatozoa from some of these infertility patients exhibited acrosomal exocytosis after ionophore exposure. Thus use of ionophore, but not mannose or progesterone, would result in false-positive findings.

These data suggested that category 3 patient spermatozoa were coordinately depleted of receptor proteins capable of

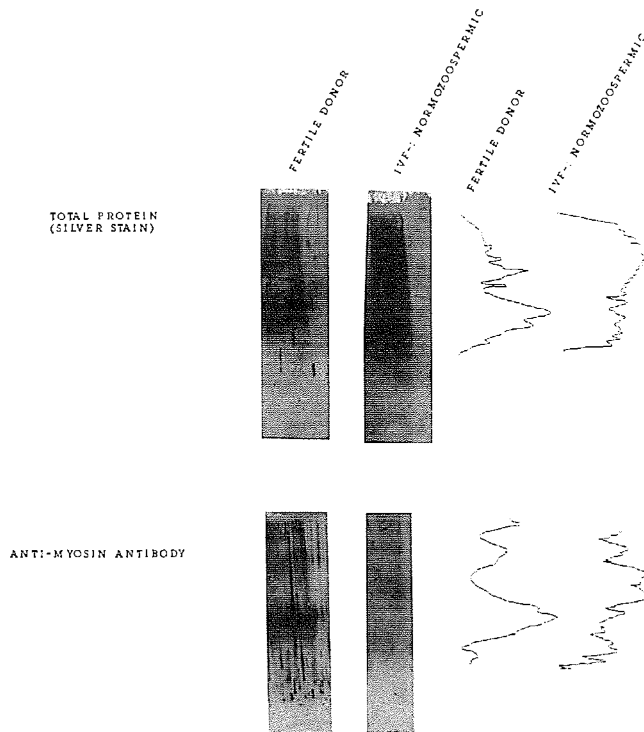

Figure 7. Comparison of protein species reactive with anti-myosin antibodies in total proteins extracted from a fertile donor versus from a category 3a male, a normozoospermic in-vitro fertilization (IVF) failure with unexplained infertility. Equal numbers of cell equivalents (0.5×10^6) were loaded in each lane. Paired photographs of Western blots are shown. Corresponding densitometric tracings were prepared from photographic negatives (Polaroid 665 film). (Upper panels) Total protein was detected using silver-enhanced colloidal gold staining. (Lower panels) Extracts from fertile donor spermatozoa display the full complement of protein species reactive with anti-myosin antibodies (Benoff *et al.*, 1994a). In contrast, spermatozoa from the category 3a normozoospermic IVF failures were specifically depleted in proteins containing myosin-like motors.

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.*, 1993b,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 iatrogenic infertility associated with therapeutic administration of calcium channel blockers (Benoff *et al.*, 1994b, 1995b; Herschlag *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

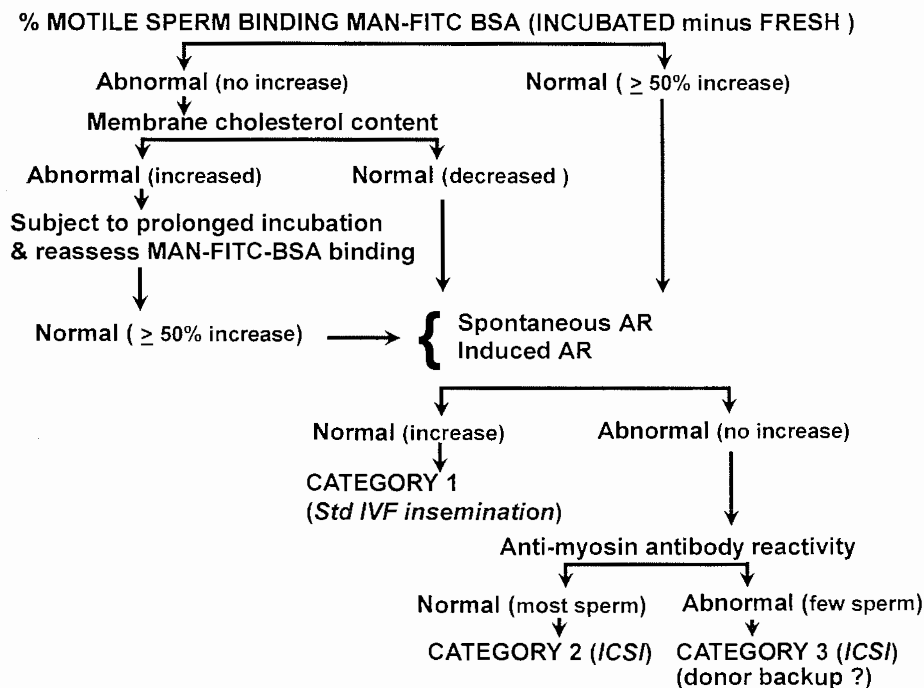

Figure 8. Flow chart representing the order of methodology implementation and the potential outcome.

believe that ionophores are of limited value for male fertility evaluation. In contrast, micromolar quantities of polyvalent mannose-ligands and millimolar amounts of mannose monosaccharide 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.*, 1995c). 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 mannose 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 *et al.*, 1993d, 1995b; Herschlag *et al.*, 1995). As illustrated in Figures 3 and 4, such prolonged incubation is associated with increased mannose lectin expression on the sperm head surface and the acquisition of the ability to undergo a mannose-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 inseminated with the husband's spermatozoa which were preincubated for three days at room temperature prior to egg retrieval. 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 subzonal insertion (SUZI) cycle and an ICSI cycle with poor 'fertilization'. In each prior cycle, 20–30 oocytes were recovered. Conventional semen parameters showed a normozoospermic 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 mannose ligand binding and substandard 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% serum/F-10 medium prior to in-vitro insemination at 10^6/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. Frozen–thawed donor spermatozoa fertilized 10 out of 10 oocytes. Comparable 100% fertilizations with 30 h preincubated husband's spermatozoa and 5 h incubated donor spermatozoa indicate that the husband's spermatozoa behave like those of category 1 males whose previous IVF failures are related to the cholesterol 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 (Huacuja *et al.*, 1981; Padron *et al.*, 1989); and (ii) of how prolonged sperm incubation converts 'negative' 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 micromanipulation inseminations.

In category 2, the percentages of motile incubated spermatozoa expressing mannose binding sites on their heads are equivalent to that observed for fertile donors. However, in contrast to the latter, category 2 spermatozoa do not undergo an acrosome reaction stimulated by polyvalent mannose ligands + D-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

zona entry characteristics have been previously described (e.g. Overstreet *et al.*, 1980; Franken *et al.*, 1993; 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 (Thomas and Meizel, 1988) required for exocytosis (Liu and Baker, 1994b). We believe that our category 2 patients represent a class 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 defect described as category 2 occurs prior to calcium influx into the spermatozoa, as polyvalent ligands are unable to effectively crosslink mannose receptors and induce aggregation, and because, importantly, there was no associated loss of acrosome content in the small percentage of spermatozoa in which mannose receptors had migrated to the equatorial segment (Benoff *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. nifedipine (Benoff *et al.*, 1994b, 1995b,e; Herschlag *et al.*, 1995), where we attributed the reduced 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 varicocele. 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 (Naftulin *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 Howards, 1987; Goldstein *et al.*, 1992; Marmar *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 (Benoff *et al.*, 1995a), we suggest that the hypothesized defect 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 cadmium are elevated and those of zinc decreased in infertile men with varicocele (Benoff *et al.*, 1996). As quantitative molecular differences in expression of integral sperm plasma membrane proteins occur among infertile men with varicocele (Benoff *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 12 oocytes being fertilized) following failure to fertilize by standard insemination. Screening by the methodology described herein prior to the commencement of an IVF cycle will direct assignment of such patients to ICSI and markedly improve chances for fertilization over that obtained with standard IVF insemination.

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 IVF 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 male had $>79\%$ tapering head forms. In the first repeat IVF cycle, none of the three oocytes were fertilized with the husband's spermatozoa following standard IVF insemination with 600 000 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 1.5×10^6 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 back-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 integral membrane proteins whose cytoplasmic domains all contain myosin-like motor sequences, i.e. actin binding and ATP nucleotide binding/ATPase activity sites (Benoff *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.*, 1993; Schweibert *et al.*, 1994) or by interaction with components of second messenger systems (Mills and Mandel, 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 glycosylated adhesion molecules containing binding sites that interact with a submembrane core protein (Goodwin *et al.*, 1990). This interaction activates the tethering and coalescence of these low affinity receptor proteins by the cytoskeleton into a high affinity junctional complex (Goodwin *et al.*, 1990; Mathur *et al.*, 1994). The human sperm mannose receptor also possesses amino acid sequences that permit its interaction with an underlying cytoskeleton (Benoff *et al.*, 1994a). Ligand stimulated mannose receptor aggregation is normally associated with acrosome loss by human spermatozoa (Benoff *et al.*, 1995e), 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/spectrin cytoskeleton 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 (Zaneveld *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; Muallem *et al.*, 1995). Cytoskeletal elements apparently participate in antigen redistribution associated with the acrosome reaction (Feuchter *et al.*, 1986; Ochs *et al.*, 1986) and in experimental models for environmental associated infertility (Benoff *et al.*, 1995d, 1996; S.Benoff and M.Barcia, 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 spontanteous 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 an absolute requirement for acrosome exocytosis, and explains how the observed depletion in category 3 spermatozoa 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 Silber'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 IVF with embryo 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.

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