Induction of the human sperm acrosome reaction with mannose-containing neoglycoprotein ligands*

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In the interest of classifying cases of male factor infertility, we have paid particular attention to the sugar ligand binding properties of the human sperm surface and the functional capacity of the acrosome for exocytosis—key parameters for assessing sperm fertilizing ability. Zona recognition and binding involve the interactions of sperm surface mannose receptors (lectins) with mannose ligands on the zona pellucida. Sperm surface mannose lectins can be visualized by their ability to bind a synthetic model zona ligand, fluorescein isothiocyanate (FITC)-conjugated mannosylated bovine serum albumin (BSA) (Man–FITC–BSA). We now report that Man–FITC–BSA biologically also mimics the effects of solubilized authentic human zonae, in that binding of Man–FITC–BSA results in a time-dependent receptor aggregation and the induction of acrosome exocytosis in capacitated sperm populations from fertile donors. In our assay, the addition of mM amounts of mannose monosaccharide to Man–FITC–BSA increases the number of polyvalent mannose ligands bound by individual spermatozoa and increases the rate of the acrosome reactions induced by Man–FITC–BSA, thereby increasing specimen processing efficiency. We conclude that exposure of human spermatozoa to polyvalent mannose ligands + D-mannose monosaccharide offers a new, convenient and readily available system to study sperm capacity for induced acrosome loss.

Key words: induced acrosome loss/mannose receptor aggregation/monosaccharides/sperm–oocyte binding/zona pellucida oligosaccharides

Introduction

The acrosome reaction is a specialized form of exocytosis required for sperm penetration through the zona pellucida (Overstreet and Hembree, 1976; Chen and Sathanathan, 1986; Liu and Baker, 1993, 1994a,b, 1996b). The elucidation of the cellular mechanisms inducing the acrosome reaction is of particular relevance to the clinical evaluation of sperm function. Human spermatozoa which are unable to undergo an acrosome reaction will not fertilize oocytes following conventional insemination in vitro (Overstreet et al., 1980; Liu et al., 1989a,b; Liu and Baker, 1994b; Benoff et al., 1996). The goal of the present study is to identify characteristics of human sperm functionally important in acrosome exocytosis. These characteristics should help classify cases of male factor infertility and, thus, could be used to predict the fertilizing potential of sperm populations.

We have concentrated on the elucidation of the properties of motile human spermatozoa able to acrosome react in response to external stimuli for two reasons. Firstly, the spontaneous acrosome reaction has been considered to be largely artifactual as in specimens of unknown fertility status it was not affected by the presence of extracellular calcium or low temperature (White et al., 1990). However, the ability of human spermatozoa to undergo an induced acrosome reaction is correlated with in-vitro fertilization (IVF) outcome (Henkel et al., 1993; Calvo et al., 1994; Parinaud et al., 1995; Benoff et al., 1996). Secondly, although both acrosome-intact and acrosome-reacted human spermatozoa can initiate binding to the human zona pellucida (Morales et al., 1989), only acrosome-reacted spermatozoa have been observed to penetrate deep through the zona (Sathanathan et al., 1982). A series of studies have now indicated that the physiologically relevant acrosome reaction of human spermatozoa occurs at the surface of the zona pellucida (Cross et al., 1988; Tesarik et al., 1988; Tesarik, 1989; Coddington et al., 1990; Liu and Baker, 1990, 1994a, 1996a,b; Franken et al., 1991; Hoshi et al., 1993). We postulated that the characterization of the acrosome reaction-inducing residues in the human zona pellucida could lead to the development of a new tool for the evaluation of sperm function prior to IVF.

All mammalian species so far tested express three protein families which make up the zona pellucida (Harris et al., 1994). There is limited evidence that human spermatozoa can

directly bind to zona polypeptides (Whitmarsh et al., 1996), that antibodies to a porcine zona protein epitope block binding of human spermatozoa to homologous oocytes (Koyama et al., 1996) and that abnormalities in the polypeptide backbone of the zona’s sperm receptor may be contributory to fertilization failure in vitro (Oehninger et al., 1996). It has, however, been inferred that it is the species-specific glycosylation of the zona proteins, and not the species-specific variation in protein sequence, which is the primary regulator of sperm–zona binding (Florman and Wassarman, 1985; Yurewicz et al., 1991; Litscher et al., 1995; Benoff, 1997). This inference is supported by lectin binding studies which indicate that the distribution of carbohydrates on human and animal zona differ significantly (Shalgi et al., 1991; Maymon et al., 1994) and also helps explain why human spermatozoa have limited affinities for heterologous zonae (Bedford, 1977; Liu et al., 1991; Oehninger et al., 1993). In contrast, the acrosome reaction-inducing activity of soluble zona glycoproteins depends both on their polypeptide conformation and glycosylation patterns (Florman et al., 1984; Litscher and Wassarman, 1996; Liu and Baker, 1996a), possibly as the protein presents to the sperm-specific oligosaccharide side chains in the form of polyvalent ligands (Leyton and Saling, 1989).

Induction of human sperm acrosome exocytosis by oligosaccharide ligands on the zona surface appears to be a complex phenomenon. Nevertheless, it is clear that sperm surface sugar receptors are involved both in the initiation of acrosome exocytosis (e.g. N-acetylgalactosamine; Brandelli et al., 1994, 1995) and its prevention (e.g. fructose; Mori et al., 1993). The best studied human sperm receptors for carbohydrates are those for mannose ligands (Mori et al., 1989; Tesarik et al., 1991; Benoff et al., 1992, 1993a, 1994a,b, 1995a,b, 1996; Chen et al., 1995). By cloning and sequencing of the human sperm surface mannose receptor, we have confirmed its homology with other human calcium-dependent (C-type) mannose-binding lectins (Benoff et al., 1992, 1993e, 1994a). Based on two deduced amino acid sequences, the sperm mannose receptor is inferred to be a heterodimer consisting of two polypeptide chains, each having two mannose carbohydrate recognition domains. We have previously used this information to design the protocols for the examination of sugar-induced acrosomal exocytosis (Benoff et al., 1993c,d, 1995a, 1996). In our prior studies we have used a brief (15 min) exposure of capacitated human spermatozoa to polyvalent neoglycoprotein ligands and monosaccharides to mimic the effects of sugar epitopes on the zona surface which induce acrosomal loss. In this study, we examined: (i) the concordance between mannose receptor aggregation by mannose or antibodies to mannose lectins and induction of the acrosome reaction; (ii) the time course of acrosome exocytosis induced by polyvalent mannose-containing neoglycoproteins in the presence or absence of monosaccharides; and (iii) the effect of D-mannose on the numbers of polyvalent neoglycoprotein ligands bound by each spermatozoon. The results of these studies have been informative for developing an understanding of how polyvalent mannose ligands and monosaccharide mannose can interact to influence ligand binding affinities and the rate of acrosome loss.

Materials and methods

Media and chemicals

Modified Ham’s F-10 medium (Formula No. 90–8050PG) was obtained from Gibco Laboratories (Grand Island, NY, 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. 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.

Semen preparation

Fresh semen specimens were collected by masturbation after 2–3 days of abstinence. Semen analysis was performed manually following liquefaction. Sperm concentration, percentage motility and progression were assessed (WHO, 1992). Spermatozoa were then selected for motility by a ‘swim-up’ method (Bronson et al., 1982). The number of spermatozoa/ml was determined by haemacytometer count after 1:20 dilution in counting fluid containing 2% phenol. Motility in swim-up preparations ranged from 97 to 100%.

Untreated (‘fresh’ or uncapacitated) spermatozoa were prepared for analysis by centrifugation (500 g for 8 min) to concentrate 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% CO2 in air. At the end of incubation, spermatozoa were collected by centrifugation and their motility was assessed by phase-contrast microscopy. Only preparations with >80% motile spermatozoa were then used in the experiments reported here.

Spermatozoa and oocytes are generally co-incubated for a standard 16–20 h period as this time frame corresponds to the timing for observation of the formation of pronuclei (Trounson and Gardner, 1993; Gianaroli et al., 1996). A similar 16–20 h incubation period for assessment of sperm function was chosen for two practical reasons. Firstly, fertile donor human spermatozoa exhibit a time-dependent increase in surface mannose receptor expression which reaches plateau levels by 18 h of incubation (Benoff et al., 1993b, 1996). Thus, this incubation period maximizes our ability to distinguish between fertile and infertile sperm populations (Benoff et al., 1993c, 1996). Secondly, mannose receptor expression is observed to rise starting at ~4 h of incubation (Benoff et al., 1993b). As a result of patient scheduling, the time required to prepare the fresh sample and to complete a surface labelling, severe constraints would be placed on clinical laboratory personnel if they were to be required to perform two mannose receptor assays on the same sample in a single day. The prolonged incubation period enables routine use of this protocol in clinical diagnosis.

Visualization of sperm surface D-mannose binding sites

To study the distribution of mannose binding sites on freshly isolated and capacitated human spermatozoa, motile sperm populations were surface labelled with 100 µg/ml fluorescein isothiocyanate-conjugated mannosylated bovine serum albumin [Man–FITC–BSA; a neoglycoprotein prepared by reaction of a mannose-containing glycosidophenyl isothiocyanate with BSA (Monsigny et al., 1994; Sigma No. A7790)] as previously described (Benoff et al., 1993b). Control reactions contained 100 µg/ml FITC-conjugated BSA. In some experiments
Man–FITC–BSA was replaced with polyclonal goat serum prepared against the human macrophage mannose receptor (anti-HMR antibodies; a generous gift of Dr Philip D.Stahl, Washington University, St Louis, MO, USA), diluted 1:1000 in Ca$^{2+}$-supplemented buffer and control aliquots were treated with pre-immune goat sera (Benoff et al., 1992, 1993e). FITC-conjugated rabbit anti-goat immunoglobulin (Ig)G at 1:16 dilution served as secondary antibody. With either Man–FITC–BSA or anti-HMR antibodies, motility and viability (by eosin Y dye exclusion) were assessed at the beginning and end of the labelling protocol. In addition, in some experiments 75 mM D-mannose monosaccharide (D-mannose) or 75 mM D-galactose monosaccharide (D-galactose) was added to the Ca$^{2+}$-supplemented buffer but not to the Ca$^{2+}$-free buffer.

Man–FITC–BSA and anti-HMR antibodies display similar distributions on the human sperm head (Benoff et al., 1993b,e). Binding was enumerated as whole head plus midpiece (pattern II) or equatorial/post-equatorial regions plus midpiece (pattern III) (Figure 1A) as previously described (Benoff et al., 1993b,d, 1994b).

**Evaluation of mannose ligand binding by spectrofluorometry**

A new assay has been developed in order to quantify the intensity of fluorescence of Man–FITC–BSA molecules bound to the sperm head. Motile sperm populations were surface labelled with Man–FITC–BSA as described above. Following post-labelling washes, labelled spermatozoa were resuspended in 3 ml Ca$^{2+}$-free buffer (Benoff et al., 1993b) and the fluorescence emission quantified for 5 s in a Shimadzu RF5000U spectrofluorometer with monochromators set at 490 and 520 nm respectively, for excitation and emission. This assay is particularly useful for assessing sperm mannose-ligand receptor expression in specimens where $<1 \times 10^6$ motile spermatozoa are obtained.

**Evaluation of acrosome status**

Acrosome-intact and acrosome-reacted Man–FITC–BSA or anti-HMR antibody labelled spermatozoa were ethanol permeabilized, differentiated by reaction with 100 µg/ml rhodamine-labelled *Pisum sativum* agglutinin (RITC–PSA; Vector Laboratories Inc, Burlingame, CA, USA) in distilled water (Cross et al., 1986) and scored for the presence or absence of acrosome content (Figure 1B) as previously described (Benoff et al., 1993b, 1994b).

**Statistical analysis**

All statistical analyses were performed with the SAS/PC software package (SAS Institute Inc, Cary, NC, USA). Statistical significance was set at $P < 0.05$. In order to compare the rate of agreement between topology of Man–FITC–BSA binding (pattern II versus pattern III) and acrosomal status (intact versus reacted) in spermatozoa from fertile donors, the Kappa statistic was used. Repeated measures analyses of variance, or paired $t$-tests if only two conditions were present, were used to compare the means of the percentages of sperm binding Man–FITC–BSA or anti-HMR antibodies under various test conditions (e.g. incubation time, added monosaccharides).

The relationship between the percentage of incubated sperm binding Man–FITC–BSA (patterns II + III combined) versus relative spectrofluorometric deflection (fluorescence intensity) was examined by linear regression. The paired $t$-test was employed to compare the fluorescence intensity of aliquots of incubated spermatozoa labelled with Man–FITC–BSA in the presence or absence of 75 mM D-mannose.

**Results**

**Human spermatozoa undergo an acrosome reaction induced by polyvalent mannose-ligands**

The topology of sperm surface binding sites strictly correlates with the state of the acrosome in spermatozoa from fertile donors. Irrespective of whether Man–FITC–BSA ($n = 25$; Figure 1A) or anti-HMR antibodies ($n = 26$) were employed to detect the distribution of mannose lectins on the human sperm surface, 100% of spermatozoa exhibiting pattern II...
labelling were acrosome intact whereas 100% of spermatozoa labelled in pattern III were acrosome-reacted (Figure 1B). Kappa for each donor was 1.00, indicating complete agreement between the distribution of mannose lectins and acrosome status.

Both polyvalent mannose ligands and anti-HMR antibodies have the potential to cross-link individual mannose lectin heterodimers to one another. Thus, binding of either probe could result in a topographical redistribution of sperm surface mannose binding sites. To clarify the relationship between mannose receptors distributed in pattern II versus pattern III, we examined the effect of the time course of polyvalent mannose ligand exposure on surface distribution of mannose lectins. Motile fresh and incubated sperm populations were surface labelled under standard conditions with 26.5 μM mannose as a polyvalent ligand, Man–FITC–BSA, while varying the incubation period, e.g. 5 min, 15 min, 1 h and 2 h. A total of 25 specimens from 12 fertile semen donors were examined by this protocol and concordant results were obtained. Figure 2 presents typical results obtained by the repeated analysis of specimens (n = 13) from one fertile donor, who served as an intra- and inter-experimental control.

The Man–FITC–BSA surface labelling reaction with capacitated spermatozoa was complete within 15 min of incubation; 21.2 ± 1.9% of spermatozoa exhibited surface mannose binding sites (Figure 2A). No further increase in the percentage of spermatozoa exhibiting head-directed Man–FITC–BSA binding (pattern II + III combined) was detected upon further incubation (Figure 2A). There was, however, a significant time-dependent transition from pattern II to pattern III Man–FITC–BSA binding (Figure 2B). At 5 min of incubation, <30% of spermatozoa binding Man–FITC–BSA were labelled in pattern III whereas by 2 h of incubation, >70% of spermatozoa binding Man–FITC–BSA were labelled in pattern III.

The transition from pattern II to pattern III Man–FITC–BSA binding was accompanied by a corresponding time-dependent increase in the percentage of spermatozoa which had undergone acrosomal exocytosis as determined by double labelling with RITC-PSA. The percentages of spontaneously acrosome-reacted incubated spermatozoa in control aliquots was 19.44 ± 13.33%, consistent with prior observations (e.g. White et al., 1990; Hershlag et al., 1997). At 2 h of incubation in the presence of Man–FITC–BSA, the percentages of acrosome loss were significantly increased. Subtraction of baseline spontaneous acrosome loss from the total loss indicates that an acrosome reaction had been induced in 15.7 ± 1.52% of spermatozoa from 13 replicates from a single fertile donor (Figure 2B), a value consistent with consensus normal values of >10% for acrosome reaction inducibility (ESHRE Andrology Special Interest Group, 1996). Mean motilities and viabilities at the beginning and end of the incubation periods differed by <10%. Thus, there was no significant loss of motility or sperm viability associated with exposure to Man–FITC–BSA. These data confirm that incubated, i.e. capacitated, human spermatozoa undergo an acrosome reaction when presented with micromolar amounts of mannose as polyvalent ligand.

In contrast, when freshly isolated spermatozoa were studied, neither the percentage of spermatozoa binding Man–FITC–BSA in pattern III nor the percentage of acrosome loss increased when the surface labelling reaction time was extended from 5 min to 2 h (acrosome-reacted spermatozoa respectively, 6.0 ± 1.96% versus 5.86 ± 1.02%). These data indicate that human spermatozoa normally acquire the ability to initiate an acrosome reaction in response to mannose ligand exposure following capacitation.

**D-mannose accelerates polyvalent mannose-ligand-induced acrosome loss**

To examine whether interactions between the four carbohydrate recognition domains of the human sperm mannose receptor could change the time course of conversion of pattern II to pattern III, aliquots from the above described specimens

![Figure 2](image-url)

**Figure 2.** Examination of capacitated motile human spermatozoa for the effect of D-mannose on the conversion of fluorescein isothiocyanate-conjugated mannosylated bovine serum albumin (Man–FITC–BSA) binding pattern II to pattern III. The results of the repeated analysis of specimens (n = 13) from one fertile donor are presented. (A) Surface labelling is complete by 15 min of incubation; no increase in the percentage of head binding of Man–FITC–BSA was detected upon further incubation. The addition of D-mannose to the pre-wash and labelling buffers has no effect on the total percentage of sperm exhibiting head-directed Man–FITC–BSA binding (patterns II + III combined). For the four points examined, the P values ranged from 0.8921 to 0.9673 (not significant). (B) Exposure of spermatozoa to polyvalent mannose ligands (100 μg/ml Man–FITC–BSA containing 26.5 μM mannose) over time induces the transition from pattern II to pattern III Man–FITC–BSA binding (P <0.006). The addition of D-mannose significantly increases the rate at which the pattern II to III conversion occurs (5 min, P <0.0061; 15 min, P <0.0001; 60 min, P <0.0045). This transition is complete by 2 h of exposure (0 versus 75 mM D-mannose; P = 0.967, not significant) irrespective of whether or not D-mannose was added.
(n = 25) were surface labelled with Man–FITC–BSA in the presence of d-mannose for 5 min, 15 min, 1 h or 2 h (Figure 2). d-mannose was added at 75 mM because including this concentration of free sugar in the pre-wash and labelling reaction buffers had no effect on the total percentage of spermatozoa displaying head-directed Man–FITC–BSA binding (patterns II + III combined) (P = 0.95, not significant). (B) The addition of d-mannose to the blocking and labelling buffers, however, stimulated the conversion of anti-HMR antibody binding pattern II to binding pattern III (P < 0.01).

The addition of d-mannose significantly increased the rate at which the transition from pattern II to pattern III was detected by exposure to Man–FITC–BSA (Figure 2B). These data suggest that prior exposure of human spermatozoa to d-mannose ‘primed’ or augments ligand binding when capacitated spermatozoa are subsequently exposed to Man–FITC–BSA. The enhancing effect of the added monosaccharide on the pattern III percentage was apparent even at the shortest exposure time of 5 min (polyvalent mannose-ligand alone, range: 21.5–27.6% versus polyvalent mannose-ligand + d-mannose monosaccharide, range: 42.9–48.9%). However, to ensure that all available mannose binding sites have the potential to participate in this conversion, we prefer a 15 min labelling incubation (see Figure 2A).

Irrespective of whether or not the buffers included d-mannose, the pattern II to pattern III transition was complete by 2 h of incubation. Importantly, the addition of d-mannose to Man–FITC–BSA did not increase the final percentage of spermatozoa exhibiting an acrosome reaction induced by zona ligands (Benoff et al., 1995b, 1996).

Freshly isolated spermatozoa could not be stimulated to undergo the pattern II to pattern III transition by exposure to Man–FITC–BSA + d-mannose, nor did such exposure increase the level of acrosome loss in that small percentage of motile spermatozoa capable of binding Man–FITC–BSA immediately after a 1 h swim-up. There was no difference between results obtained using aliquots surface labelled for only 5 min and duplicate aliquots labelled up to 2 h (control versus + d-mannose at 5 min respectively, 6.0 ± 1.96% versus 4.71 ± 1.51%; at 2 h respectively, 5.86 ± 1.02% versus 6.09 ± 1.73%). These findings provide further support for the hypothesis that the ability to undergo an induced acrosome reaction is acquired during capacitating incubations.

To show that the d-mannose could stimulate acrosomal exocytosis triggered by another polyvalent species capable of cross-linking mannose lectins, the effect of d-mannose on the topology of anti-HMR antibody binding was determined. All aliquots were exposed to anti-HMR antibodies under standard incubation conditions. Typical results are presented in Figure 3.

As observed for Man–FITC–BSA binding, the inclusion of d-mannose in the pre-wash and labelling buffers had no effect on the total percentages of spermatozoa exhibiting head-directed anti-HMR antibody binding (patterns II + III combined; Figure 3A). The addition of d-mannose to anti-HMR

**Figure 3.** Examination of the effect on capacitated motile human sperm populations from fertile donors (n = 26) of d-mannose on the conversion of pattern II to pattern III anti-human macrophage mannose receptor (anti-HMR) antibody binding. Typical results are shown. (A) The addition of d-mannose to the blocking and labelling buffers has no effect on the total percentage of spermatozoa exhibiting head-directed anti-HMR antibody binding (patterns II + III combined) (P = 0.95, not significant). (B) The addition of d-mannose to the blocking and labelling buffers, however, stimulated the conversion of anti-HMR antibody binding pattern II to binding pattern III (P < 0.01).

**Figure 4.** Examination of the ability of different monosaccharides to prime the acrosome reaction initiated by polyvalent mannose ligands. The results of analysis of specimens from four fertile donors are presented. At all time points, there is a significant difference between the three conditions (no monosaccharide added versus +d-galactose versus +d-mannose, range of P values: 0.0004–0.0055) on the percentage of spermatozoa binding fluorescein isothiocyanate-conjugated mannosylated bovine serum albumin (Man–FITC–BSA) in pattern III as a fraction of total binding (patterns II + III combined). No difference was detected between no monosaccharide added versus +d-galactose (P: 0.0095–0.566, not significant). In contrast, the addition of d-mannose dramatically increased the subpopulation (no monosaccharide added versus +d-mannose, P: 0.0069–0.0407; +d-galactose versus +d-mannose, P: 0.0051–0.0267).
The density of fluorescein isothiocyanate-conjugated mannosylated bovine serum albumin (Man–FITC–BSA) binding on the human sperm head examined by spectrofluorometry: (A) The determination of the fluorescence intensities of serial 1:2 dilutions of motile spermatozoa from fertile donors (n = 6) surface labelled with Man–FITC–BSA indicates that a linear relationship between cell number and relative spectrofluorometric deflection of both freshly isolated, untreated human spermatozoa (Pearson correlation coefficient, r = 0.998, P <0.0001) and duplicate aliquots subjected to capacitating conditions (Pearson correlation coefficient, r = 0.949, P <0.0038). Furthermore, spectrofluorometry readily illustrates the increase in Man–FITC–BSA binding which occurs following capacitation. (B) Unlabelled incubated sperm and incubated sperm surface labelled with Man–FITC–BSA from fertile donors (n = 6) were mixed to give varying percentages of spermatozoa binding Man–FITC–BSA (patterns II + III) in a total number of 0.25×10^6 spermatozoa. A linear relationship was detected (Pearson correlation coefficient, r = 0.937, P <0.0006). (C) Spectrofluorometric analysis of motile sperm from fertile donors (n = 3) surface labelled with Man–FITC–BSA in the presence of D-mannose versus control aliquots labelled with Man–FITC–BSA in the absence of added monosaccharide indicates that the fluorescence intensity of monosaccharide-treated aliquots is increased by an average of 48.5 ± 3.5% (P <0.0089).

antibodies stimulated the conversion of pattern II to pattern III binding (Figure 3B), again as observed with Man–FITC–BSA. A simultaneous increase in acrosomal exocytosis was also detected in specimens double-labelled with RITC-PSA (not shown).

To examine whether monosaccharides other than D-mannose could prime or stimulate the acrosome reaction induced by polyvalent mannose ligands, aliquots of motile capacitated spermatozoa populations from fertile donors (n = 4) were preincubated in buffers supplemented with D-galactose or D-mannose. Substitution of D-galactose for D-mannose did not alter the osmolality (390 mOsm) of the buffer. Preincubations proceeded for 5 min, 15 min or 1 h prior to a 15 min surface labelling with Man–FITC–BSA. Control aliquots were incubated in Ca^2+-supplemented core buffer without any added monosaccharide (Figure 4). Pre-exposure of human spermatozoa to D-galactose did not change the percentage of spermatozoa exhibiting head-directed Man–FITC–BSA binding (patterns II + III combined), confirming prior observations (Benoff et al., 1993d). More importantly, exposure of incubated spermatozoa to D-galactose had no effect on the distribution of mannose-ligand binding sites nor did it affect acrosome
status. There was no stimulation of the transition of pattern II to pattern III Man–FITC–BSA binding and there was no increase in loss of acrosome content.

The human sperm acrosome reaction has been reported to be influenced by the osmolality of the external medium, and hyper-osmolar conditions (>449 mOsm) can inhibit ionphore-induced acrosome loss (Bielfeld et al., 1993). However, the osmolality of the D-galactose-containing buffer cannot account for the failure of D-galactose to stimulate acrosome loss initiated by mannose-containing neoglycoprotein binding. These data indicate that the priming effect upon acrosomal exocytosis resulting from pre-exposure of spermatozoa to monosaccharides is specific to the structure of the sugar.

**Allosteric interactions between mannose carbohydrate recognition domains contribute to acrosome loss**

That polyvalent anti-HMR antibodies could more rapidly cross-link mannose lectins in the presence of D-mannose suggested that the number of antigenic epitopes on the sperm surface were increased by sugar exposure. In addition, we noted that the intensity of sperm surface labelling with Man–FITC–BSA was markedly increased when D-mannose was added to the buffers employed in the pre-wash and labelling reactions, confirming our prior observations (Benoff et al., 1993c,d). To determine if these effects were due to an increase in mannose binding sites, we have assayed the intensity of fluorescence of Man–FITC–BSA as determined by microscopic inspection of stained slides (Figure 5B). No apparent artifacts are detected by varying the number of cells analysed (Figure 5A).

In the second control series, unlabelled sperm populations were mixed with Man–FITC–BSA surface labelled populations up to a combined total number of 0.25×10⁶ spermatozoa. The resultant percentage of spermatozoa exhibiting head-directed Man–FITC–BSA binding was determined by microscopic inspection. In all samples tested (n = 6), fluorescence emission was proportional to the percentage of spermatozoa binding Man–FITC–BSA as determined by microscopic inspection of stained slides (Figure 5B).

This assay was then employed to examine the relative fluorescence intensity of incubated sperm surface labelled with Man–FITC–BSA in the presence of D-mannose versus control aliquots (n = 3) (Figure 5C). The fluorescence emission of D-mannose-treated aliquots increased by an average of 48.5 ± 3.5% at all cell concentrations tested. Thus, in the presence of non-competing levels of D-mannose which leaves unchanged the total number of spermatozoa binding Man–FITC–BSA on their surface, the number of Man–FITC–BSA molecules bound by individual spermatozoa is increased. This increased fluorescence emission detected fluorometrically supports the notion that allosteric interactions are occurring between multiple mannose binding sites on the same heterodimeric sperm surface lectin, making more binding sites on each spermatozoa accessible to polyvalent mannose ligands.

**Discussion**

The ESHRE Andrology Special Interest Group (1996) reviewed four areas of advanced diagnostic andrology previously referred to as ‘research tests’. The recommendations for standardization of sperm function testing are of particular relevance to the current study. Consensus points included elimination of the analysis of the spontaneous acrosome reaction as a direct diagnostic test or in the zona-free hamster oocyte penetration test. Rather, it was concluded that both clinical tests be performed with sperm population induced to acrosome react with the ionophore A23187. However, because ionophores bypass membrane mechanisms which regulate calcium influx into spermatozoa (e.g. Goodwin et al., 1997), false positive results can often be obtained (Benoff et al., 1996).

During this ESHRE meeting, Tesarik suggested that recombinant human zona pellucida glycoproteins (rhuZP) will serve as agonist in the ‘perfect acrosome reaction test’ (ESHRE Andrology Special Interest Group, 1996). After prolonged incubation the unglycosylated polypeptide backbone of rhuZP synthesized in a coupled in-vitro transcription/translation system is capable of inducing the human sperm acrosome reaction (Whitmarsh et al., 1996). However, glycosylation of rhuZP plays an important role in induction of the acrosome reaction (Barratt et al., 1994; Van Duin et al., 1994) and rhuZP synthesized in cell lines capable of fully glycosylating the expressed protein rapidly induces high levels of acrosome loss (Brewis et al., 1996). Large scale production of a biologically active rhuZP, required for routine use in clinical assessment of the ability of spermatozoa to undergo an acrosome reaction, is not currently feasible (Whitmarsh et al., 1996). In this report we demonstrate that polyvalent mannose ligands and monosaccharide mannose serve as a cost effective alternative for rhuZP that can be readily and immediately applied by many laboratories.

It is now generally agreed that the acrosome reaction is a receptor-mediated event (ESHRE Andrology Special Interest Group, 1996). It is therefore significant that the structure of the human sperm mannose-ligand receptor has at least two features critical for its interaction with the zona pellucida and induction of the acrosome reaction. First, the active site of the human sperm mannose-ligand receptor is a C-type lectin, sharing a common sugar recognition motif with a large family of similar molecules (Drickamer, 1989). Ca²⁺-dependent mannose-specific lectins of human somatic cells have been reported to be ‘core-specific’, i.e. recognizing not only the terminal sugar but also those within the core region of the oligosaccharide (Summerfield and Taylor, 1986). These observations suggest that mannose need not be the terminal residue of zona glycoconjogates for sperm mannose lectins to serve as zona receptors. Second, both components of the human sperm

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mannose-ligand receptor dimer contain two carbohydrate recognition domains. This duplication suggested that mechanisms for sperm recognition of zona pellucida mannose residues rely on cooperative interactions between multiple mannose recognition domains, which individually have a weak affinity for monosaccharides. Competition experiments have demonstrated that the human sperm mannose receptor has only low affinity for D-mannose monosaccharide. A 10 000-fold excess monosaccharide as compared with D-mannose linked to BSA was required to achieve equivalent (>70%) inhibition of Man–FITC–BSA binding (Benoff et al., 1993d). Similar findings have been reported for other animal lectins and their corresponding ligands (e.g. Lee and Lee, 1987; Lee et al., 1992; Taylor et al., 1992; Taylor and Drickamer, 1993) and for the purification of the lectins by affinity chromatography (Lennartz et al., 1987). These structural features were the background for our experiments.

The present study was prompted by our observation that exposure of capacitated motile sperm populations from fertile donors to polyvalent mannose ligands and D-mannose for only 15 min markedly increased the relative percentages of spermatozoa binding Man–FITC–BSA in pattern III (Benoff et al., 1993c,d). As observed in animal models (Leyton and Saling, 1989; Beebe et al., 1992), ligand-initiated receptor aggregation appears to be an early step in the induction of the human sperm acrosome reaction. In our fertile donors, there was a 1:1 concordance between sperm binding Man–FITC–BSA in the equatorial/post-acrosomal segment of the head (pattern III) and spermatozoa which had undergone acrosomal loss. This direct correspondence had hitherto been implied rather than demonstrated in our previous publications (Benoff et al., 1993c,d). Clearance of the acrosomal plasma membrane of integral membrane proteins facilitates acrosomal exocytosis. However, in the spermatozoa from infertile men where Ca\(^{2+}\) influx into spermatozoa is blocked by anti-hypertension medications, we consistently find that pattern III Man–FITC–BSA binding occurs on acrosome-intact spermatozoa, i.e. in the absence of an acrosome reaction (Benoff et al., 1994b, 1995a; Hershlag et al., 1995; Goodwin et al., 1997). These data indicate that Ca\(^{2+}\) influx (Thomas and Meizel, 1988) is not obligatory for mannose receptor movement between contiguous regions of the plasma membrane. These observations also demonstrate that mannose lectin translocation seems to be required for the induction of an acrosome reaction, but does not seem to be the only determinant.

We report that binding of polyvalent mannose ligands appears to induce an allosteric change in either the mannose lectin or in some super-molecular complex of which mannose lectin forms a part (e.g. with the non-nuclear progesterone receptor; Benoff et al., 1995b). We also report that the monosaccharide and the polyvalent form of mannose together increase the rate at which the conformational change occurs in mannose lectins on the sperm surface. In either case, the allosteric change exposes additional mannose binding sites. Similar observations have been made in related systems (Lee and Lee, 1987; Taylor et al., 1992; Weis et al., 1992). Interaction between multiple carbohydrate recognition domains markedly contributes to high affinity binding of polyvalent ligands to other mammalian mannose-specific lectins (Lee et al., 1992; Taylor et al., 1992). These observations support our conjecture based on partial primary sequences of the mannose receptor that binding of mannose by one carbohydrate recognition domain results in a change in the accessibility of the remaining carbohydrate recognition domains to external ligands. More importantly, this mechanism is consistent with data on the kinetics of the human sperm acrosome reaction induced by increasing doses of solubilized human zona pellucida glycoproteins (Henkel et al., 1995) wherein a sigmoidal curve was obtained. The shape of the curve provides evidence of cooperativity and indicates that human spermatozoa must bind multiple zona ligands in order for exocytosis to occur.

The acrosome reaction stimulated by mannose-containing neoglycoproteins shares five additional characteristics with the zona pellucida-induced acrosome reaction: (i) induction of the acrosome reaction by polyvalent mannose ligands requires prior capacitation of sperm populations. Human spermatozoa acquire the ability to acrosome react in response to intact or solubilized human zonae following 6–8 h capacitation in vitro (Overstreet and Hembree, 1976; Hoshi et al., 1993). Mannose lectins appear on the sperm surface with a similar time course (Benoff et al., 1993b); (ii) preliminary studies suggest that after mannose receptors have redistributed to the equatorial/post-acrosomal region of the sperm head, signal transduction proceeds via activation of protein phosphokinases (Benoff, 1997; A.Jacob and S.Benoff, unpublished observations) and Ca\(^{2+}\) influx through a voltage-dependent Ca\(^{2+}\) channel (Goodwin et al., 1997; Jacob et al., 1997). Interaction at fertilization of a human sperm tyrosine kinase with the zona pellucida has previously been reported (Burks et al., 1995) and activation of voltage-dependent Ca\(^{2+}\) channels is required for zona pellucida-induced acrosomal exocytosis (Florman et al., 1992; Florman, 1994); (iii) the time course of the mannose-initiated acrosome reaction, wherein maximum percentages of acrosomal exocytosis are observed by 2 h of incubation, is consistent with ultrastructural reports of acrosome loss and sperm penetration into intact zonae (McMaster et al., 1978; Sathananthan et al., 1982; Chen and Sathananthan, 1986; Cross et al., 1988; Liu and Baker, 1994a); (iv) we have shared our protocols for oligosaccharide-stimulated induction of the acrosome reaction with others who independently have confirmed these results (Amin et al., 1995). Stages in the zona pellucida-mediated acrosome reaction may be monitored using a chlorotetracycline (CTC) fluorescence assay (Lee et al., 1987; Kligman et al., 1991). The CTC and mannose methods were compared and found to produce equivalent results: both loss of CTC fluorescence over the sperm head and the pattern II to pattern III Man–FITC–BSA binding transition reflect the formation of hybrid vesicles of the plasma and outer acrosomal membranes, normally observed by electron microscopy (e.g. Chen and Sathananthan, 1986); (v) the maximum percentages of spermatozoa undergoing acrosome loss following mannose challenge are similar to those obtained with solubilized zonae.
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