Role of monoclonal antibody J-23 in inducing acrosome reactions in capacitated mouse spermatozoa

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Summary. A monoclonal antibody (J-23) to the 15 kDa component on the sperm head, the acceptor, which functions in zona binding, was shown to induce the acrosome reaction in capacitated cells, but not in fresh cells. The antibody recognized its epitope in the acrosomal cap region of fresh spermatozoa and in the equatorial region on washed and capacitated spermatozoa. However, equatorial expression did not depend on the acrosome reaction, since washing fresh spermatozoa increased the percentage with equatorial fluorescence, but did not increase the percentage with reacted acrosomes. The data indicate that the acrosome reaction can be induced in capacitated spermatozoa in the absence of zona glycoproteins.

Keywords: spermatozoa; acrosome reaction; capacitation; mouse

Introduction

ZP3, one of the three glycoproteins that make up the murine zona pellucida, plays two important roles in the early events of fertilization (Wassarman, 1990a, b). It is responsible for the binding of capacitated spermatozoa to the zona and for inducing the acrosome reaction in the bound cells. With the acrosome reaction, spermatozoa can penetrate the zona and fuse with the egg vitellus (Bleil & Wassarman, 1980a, b, 1983; Wassarman *et al.*, 1985). Binding to the zona depends in part on the ability of the spermatozoa to recognize a galactose residue on a class of O-linked oligosaccharides of ZP3 (Florman & Wassarman, 1985; Bleil & Wassarman, 1988). However, the protein moiety of this complex molecule is necessary for inducing the acrosome reaction (Bleil & Wassarman, 1983; Wassarman *et al.*, 1985). The trigger for the zona-induced acrosome reaction appears to be the aggregation of zona-binding sites on the head of capacitated spermatozoa (Leyton & Saling, 1989a; Wassarman, 1990a). This suggestion is based on the following observations. Zona glycopeptides, produced by pronase treatment, bind to the sperm surface and inhibit sperm–zona binding but cannot induce the acrosome reaction. Aggregation of the bound glycopeptides with antibodies to ZP3 induces acrosome reactions as efficiently as solubilized whole zonae or isolated ZP3 (Leyton & Saling, 1989a). These results suggest that immuno-aggregation of zona binding sites, in the absence of zonae components, would result in the acrosome reaction (Saling, 1989).

A number of molecules from spermatozoa have been implicated in sperm–zona binding (Saling, 1989 for review). These include a 95 kDa plasma membrane protein (Leyton & Saling, 1989b), galactosyltransferase (Shur & Hall, 1982) and a trypsin-inhibitor sensitive site (Saling, 1981; Poirier *et al.*, 1986; Benau & Storey, 1987). The trypsin-inhibitor-sensitive site was defined by blocking sperm–zona binding with a variety of synthetic and natural proteinase inhibitors (Saling, 1981). The male reproductive tract is a rich source of such inhibitors (Haendle *et al.*, 1965). In the mouse a 6.4 kDa proteinase inhibitor from the seminal vesicle binds to the acrosomal cap region at ejaculation (Poirier & Jackson, 1981; Irwin *et al.*, 1983) and is removed during *in utero* and *in vitro*

incubation (Robinson *et al.*, 1987; Boettger *et al.*, 1989). The inhibitor binding site, the acceptor, on the sperm head has been purified by affinity chromatography using the inhibitor as the ligand (Aarons *et al.*, 1984). Treating cumulus-free, zona-intact oocytes with the purified acceptor reduces, in a concentration-dependent fashion, the number of spermatozoa that can bind (Poirier *et al.*, 1986). A monoclonal antibody (J-23) to the site has been prepared and characterized (Richardson *et al.*, 1987). Since the site functions in zona binding, its antibody may by immunoaggregation be effective in induction of the acrosome reaction.

Some sperm surface components that function in zona binding are rearranged over the sperm head with the acrosome reaction (Lopez & Shur, 1987; Myles & Primakoff, 1984). These modifications may play a role in holding acrosome reacted spermatozoa to the zona during penetration. Preliminary observations (Battle *et al.*, 1990) indicate that the epitope recognized by J-23, initially visible in the acrosomal cap region on fresh cauda epididymal spermatozoa, becomes visible on the equatorial domain after 90 min incubation *in vitro*. A possible explanation for this observation is that J-23 induces the acrosome reaction in these spermatozoa and the acrosome reaction is the stimulus for the equatorial expression. The purpose of this study was to determine the role of the acrosome reaction in the expression of the equatorial epitope.

Materials and Methods

Animals

ICR mice, 8–10 weeks of age, had free access to food and water and were maintained at $22 \pm 2^\circ\text{C}$. Light was controlled on a 16 h light:8 h dark cycle.

Media

The culture medium used for the sperm incubations was Earle's modified medium 199 supplemented with 2 mg bovine serum albumin ml^{-1} and $30 \mu\text{g}$ pyruvate ml^{-1} (Bleil & Wassarman, 1980b). This medium, M-199 M, can support capacitation and fertilization.

Antibodies and antisera

The monoclonal antibody, J-23, was obtained by fusing rat spleen cells from an animal immunized with a crude acceptor preparation to a murine myeloma (S194/5XXO.BO.1) cell line. J-23 is an IgM molecule with a kappa light chain and has been fully characterized (Richardson *et al.*, 1987). J-23 recognizes whole spermatozoa, crude acceptor and purified acceptor as determined by ELISA. It binds, as shown by the western blot technique, to a single 15 kDa component, the size previously determined for the acceptor (Aarons *et al.*, 1984). J-23 used in this study was the unconcentrated hybridoma supernatant with a titre of 1:10 when used on fresh cauda epididymal spermatozoa as determined by ELISA. The titre was defined as the dilution that gave half maximum activity. The specificity of the rabbit anti-mouse seminal vesicle proteinase inhibitor has been tested by a number of techniques (Poirier & Jackson, 1981; Irwin *et al.*, 1983; Richardson *et al.*, 1987).

Two other rat monoclonal antibodies (mAbs) (M and C-9) were used as negative controls for the J-23 treatment. These antibodies were prepared in a manner similar to J-23. Both are IgM molecules as determined by an ELISA-based typing kit (Zymed, San Francisco, CA). The antibody, M (Poirier & Nicholson, 1984), recognizes an epitope on a low molecular mass (4 kDa) proteinase inhibitor found in the murine testes and caput epididymis. The epitope is present on the cap region of caput spermatozoa. The titre of the hybridoma supernatant is 1:16 as determined by ELISA using the supernatant of detergent-treated caput spermatozoa as the antigen at a protein concentration of $100 \mu\text{g ml}^{-1}$. The second rat mAb, C-9, which binds to an unknown epitope in the acrosomal cap region of cauda spermatozoa, was used to determine whether the binding of all IgM antibodies in the cap region induces acrosome reactions.

Inhibitor isolation and activity measurement

The inhibitor was isolated from the supernatant of homogenized seminal vesicle tissue by gel filtration (Sephadex G-75), affinity chromatography using trypsin as the ligand and ion-exchange chromatography (SP-Sephadex C-25) as previously described (Poirier & Jackson, 1981). Measures of inhibitory activity were determined against porcine pancreatic trypsin using *N*-benzoyl-DL-arginine-*P*-nitroanilide (BAPNA) as the substrate in Ca^{2+} -free

triethanolamine hydrochloride buffer (pH 7.8), 0.2 mol l^{-1} (Fritz *et al.*, 1974). Each 3 ml assay contained $6.0 \mu\text{g}$ trypsin, $0.77 \text{ mmol BAPNA l}^{-1}$ and various amounts of inhibitor. An inhibitor unit (IU) is defined as the amount of inhibitor that reduces the hydrolysis of BAPNA by $1 \mu\text{mol min}^{-1}$.

Indirect immunofluorescence

For acceptor localization and J-23 treatment, spermatozoa from the cauda region of the epididymis were dispersed directly into M199-M. Particulate matter was removed by filtering through two layers of Kimwipes. The spermatozoa were diluted to $5 \times 10^6 \text{ ml}$ and treated with J-23 or incubated at 37°C in a moist atmosphere of 5% CO_2 :95% air. J-23 (0.2 ml) was added to 0.1 ml of the sperm suspension. The spermatozoa were then incubated for 10 min at room temperature and washed once with phosphate-buffered saline (PBS). All incubations were performed in 1.5 ml plastic tubes. Washes were carried out in a Eppendorf model 5145 C microcentrifuge at 2500 g for 2 min. Some spermatozoa were stained with Coomassie brilliant blue. In other samples the spermatozoa were resuspended in 0.2 ml goat anti-rat IgG plus IgM, which was fluorescein labelled (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). The original antibody solution contained $30 \mu\text{g protein ml}^{-1}$ and was diluted 1:40 in PBS just before use. The suspension was incubated for 10 min at room temperature and washed twice. The spermatozoa were dried on glass slides, covered with PBS:glycerol (1:1, v/v) and observed in a Leitz Dialux microscope equipped with epifluorescence. Photographs were taken with Kodak T-Max 400 film. Controls consisted of substituting complete hybridoma medium, PBS, normal rat serum diluted 1:40 with PBS and two other rat monoclonal antibodies, M and C-9, for J-23.

For inhibitor fluorescence, sperm samples were fixed in a 1.0% solution of formaldehyde in PBS for 30 min at room temperature. The fixed samples were washed and mixed with a purified inhibitor preparation to give $30 \text{ miu } 10^{-6}$ spermatozoa. These mixtures were incubated for 15 min at room temperature and washed twice to remove unbound inhibitor. Control spermatozoa were treated with PBS and washed twice. Fc-binding sites were blocked with 3% normal goat serum for 20 min at room temperature and washed twice. The spermatozoa were then resuspended in 0.3 ml immune serum (rabbit anti-mouse seminal vesicle inhibitor) diluted 1:40 for 30 min at room temperature. The spermatozoa were then washed twice and resuspended in 0.1 ml of a 1:40 dilution of goat anti-rabbit IgG fluorescein labelled (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), for 30 min at room temperature. The undiluted sample contained $35 \mu\text{g protein ml}^{-1}$. The spermatozoa were then washed twice, resuspended in PBS and air dried to slides. Controls consisted of substituting normal rabbit serum, PBS or immune serum absorbed with the seminal inhibitor for the immune serum.

Immunodetection of antigens on nitrocellulose

Spermatozoa were collected from cauda epididymal fragments and from the ductus deferens in PBS. They were washed once and resuspended at a concentration of $3 \times 10^8 \text{ cells ml}^{-1}$ in lysis buffer for 1 h at 4°C with vortexing. The lysis buffer consisted of $50 \text{ mmol Tris l}^{-1}$ at pH 7.3, $150 \text{ mmol NaCl l}^{-1}$, 0.25% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate and 0.5% nonidet P-40. The supernatant was collected by centrifugation for 5 min in an Eppendorf minicentrifuge operating at 13000 g . Protein concentration was determined by the mini BioRad assay using bovine serum albumin as the standard. The sperm extract was mixed with an equal volume of $2 \times$ sample buffer (sample buffer = $63 \text{ mmol Tris l}^{-1}$ at pH 6.8, 1% sodium dodecyl sulfate (SDS) and 10% glycerol). Electrophoresis was performed using 13% SDS polyacrylamide gels according to the procedure of Laemmli (1970). Prestained molecular weight standards (Diversified BioTech, Newton Centre, MA) were electrophoresed in lanes adjacent to the sperm proteins. After electrophoresis, proteins were transferred to nitrocellulose ($0.45 \mu\text{m}$ pore size). The transfer buffer consisted of $26 \text{ mmol Tris l}^{-1}$, $192 \text{ mmol glycine l}^{-1}$ in 20% methanol. After the proteins were transferred, the nitrocellulose was treated with 3% non-fat instant milk in PBS for 30 min at room temperature. The nitrocellulose was then incubated for 2 h at room temperature in J-23 diluted 1:2 with PBS containing 0.5% Tween-20 (PBS-T). Control strips were incubated in 1:1000 dilution of normal rabbit serum in PBS-T. After washing (10 times for 3 min each time), the strips were incubated for 2 h in peroxidase-labelled goat anti-rat IgG plus IgM (Jackson ImmunoResearch, Inc.) diluted 1:800 in PBS-T. The sheets were washed in PBS and the peroxidase activity was detected using $1.4 \text{ mmol } 3,3\text{-diaminobenzidine tetrachloride l}^{-1}$ in PBS, pH 7.2 containing $10 \mu\text{l}$ of $30\% \text{ H}_2\text{O}_2$.

Acrosome staining using Coomassie brilliant blue

Spermatozoa were scored for acrosomes by the Coomassie brilliant blue staining technique (Moller *et al.*, 1990). Spermatozoa were fixed with 5% formaldehyde in PBS for 10 min, washed once with PBS at 5000 g and dried onto glass slides. The cells were stained for 2 min in 0.04% Coomassie G-250 made with 3.5% perchloric acid. The slides were rinsed with PBS and the spermatozoa covered with PBS:glycerol (1:1 v/v) and observed. Previous studies (Aarons *et al.*, 1991) indicate that the CB staining method which stains the acrosomal cap blue is as sensitive as the chlorotetracycline assay (Leyton & Saling, 1989a) in determining acrosome integrity after ionophore (A23187) or zona treatment.

Results

Acrosomal integrity was determined by staining with Coomassie brilliant blue. The technique is simple, requires little time and the results are unambiguous (Fig. 1).

Fig. 1. Coomassie brilliant blue stained spermatozoa. The arrows show spermatozoa without acrosomal cap staining that are considered to be acrosome reacted. $\times 1000$.

Treatment of fresh cauda epididymal spermatozoa with J-23 had little effect on the number of spermatozoa with intact acrosomes compared with untreated, PBS or the control monoclonal antibody treated samples (Fig. 2). In addition, the percentage of spermatozoa with intact acrosomes remained constant when goat anti-rat IgG plus IgM was added to J-23-treated fresh spermatozoa. However, a decrease in the number of acrosome intact spermatozoa was obtained when J-23 was added to spermatozoa previously incubated for 90 min. The addition of the goat anti-rat IgG plus IgM to these spermatozoa did not cause further increase in the percentage of acrosome-reacted spermatozoa. The decrease in the percentage of acrosome-intact spermatozoa was not observed when control monoclonal antibodies or PBS were substituted for J-23. However, the 90 min incubation reduced the number of spermatozoa with intact acrosomes by approximately 10% compared with that seen for fresh samples.

The percentage of incubated spermatozoa with intact acrosomes decreased as the amount of J-23 increased (Fig. 3). The increase had no effect on the acrosomal integrity of fresh spermatozoa.

Most fresh cauda epididymal spermatozoa express the epitope recognized by J-23 on the acrosomal cap region of the sperm head (Fig. 4). A small percentage of these spermatozoa show equatorial fluorescence. After incubation for 90 min the percentage of spermatozoa with

Fig. 2. Fresh cauda epididymal spermatozoa (■) and spermatozoa incubated for 90 min (▨) were untreated (U) or incubated in a negative control monoclonal antibody (M) or C-9 (C), PBS (P), J-23 (J) or J-23 plus goat anti-rat IgG plus IgM (J+). The data represent the percentage of spermatozoa with intact acrosomes as determined by Coomassie brilliant blue staining and are the means of seven separate trials counting 200 cells per treatment per trial. The horizontal lines represent the standard deviations of the means.

Fig. 3. One hundred microlitres of fresh cauda epididymal spermatozoa (□) or spermatozoa incubated for 90 min (△) at a concentration of $5 \times 10^6 \text{ cell ml}^{-1}$ were mixed with increasing volumes of J-23, incubated, fixed and stained with Coomassie brilliant blue. The data represent the percentage of spermatozoa with intact acrosomes and are the means of three separate trials counting 200 cells per treatment per trial. A single male was used for each trial. The horizontal lines represent the standard deviations of the means.

equatorial fluorescence has increased, whereas the percentage of spermatozoa showing fluorescence exclusively in the acrosomal cap region has decreased (Fig. 5). No fluorescence was seen in either region when normal rat serum, PBS or hybridoma supernatant was substituted for J-23. In addition C-9 bound exclusively in the acrosomal cap region on both fresh and incubated samples.

Preliminary studies (Battle *et al.*, 1990) suggested that equatorial fluorescence could be induced by washing fresh cauda spermatozoa. In an attempt to relate equatorial fluorescence to acrosome reactions, fresh spermatozoa were washed three times and the percentage with equatorial fluorescence and intact acrosomes were determined (Fig. 6). Although washing increased the percentage

Fig. 4. Indirect immunofluorescent localization of the epitope recognized by J-23 on (a) fresh cauda epididymal spermatozoa or (b) spermatozoa incubated for 90 min. $\times 800$. (c) Indirect immunofluorescent localization of the epitopes recognized by C-9 on spermatozoa incubated for 90 min. (d) A schematic representation of a mouse sperm head modified from a figure by Yanagimachi (1981) illustrating the acrosomal cap (ac), equatorial (eq) and the post-acrosomal (pa) region.

Fig. 5. Indirect immunofluorescent observations showing the percentage of spermatozoa with acrosomal cap (○), equatorial (●) or no (□) epitope expression during incubation for 90 min. The data represent the means of 20 separate trials in which 200 cells were counted for each time per trial. The horizontal lines represent the standard deviations of the means.

of spermatozoa with equatorial fluorescence, it had little effect on the percentage of spermatozoa that stained positively for intact acrosomes.

Western blot analysis of sperm components from fresh cells and cells washed and incubated for 90 min revealed a single band, in all cases, with a molecular mass of 15 kDa when probed with J-23. If the nitrocellulose was probed with PBS or normal rat serum, no band was detected (Fig. 7). Spermatozoa treated with lysis buffer no longer displayed either the acrosomal cap or equatorial epitope as determined by indirect immunofluorescence (data not shown).

Since a proteinase inhibitor from the seminal vesicle binds to the acceptor in the cap region on fresh cauda epididymal spermatozoa, we investigated whether the inhibitor would bind to the equatorial based acceptor. Spermatozoa were washed or incubated for 90 min to enhance equatorial expression and incubated with the purified seminal inhibitor. The inhibitor, localized by indirect immunofluorescence, was found only in the acrosomal cap region (Fig. 8).

Discussion

Monoclonal antibodies of the IgG class cannot usually induce aggregation unless the antigen molecule has two or more antibody binding sites (Goding, 1986). However, cross-linking can be induced in these systems with a polyvalent secondary (anti-immunoglobulin) antiserum. Monoclonal IgM molecules are more likely to cause aggregation by themselves owing to their multivalent binding sites. J-23, an IgM molecule, can induce acrosome reactions in capacitated spermatozoa in a concentration-dependent fashion. No significant increase in the percentage of acrosome-reacted spermatozoa was observed when goat anti-rat IgM plus IgG was added to J-23-treated spermatozoa. Thus the effect of J-23 is sufficient to cause a maximum response before cross-linking with a secondary antiserum. Most studies on antibody aggregation of membrane antigens use Fab fragments of the antibody as a negative control. Information concerning the proteolytic fragmentation of rat IgM is limited (Goding, 1986). Thus, it is not possible to state that J-23 causes acrosome reactions by aggregation of the epitope. IgM molecules can aggregate their antigens and the acrosome reaction does result from the aggregation of zona-binding sites (Leyton & Saling, 1989a) which suggests that J-23-induced acrosomal exocytosis could result from aggregation of the acceptor. Immunological controls included substituting two other rat monoclonal antibodies,

Fig. 6. The effects of washing fresh cauda epididymal spermatozoa on the percentage of spermatozoa with equatorial expression (E) as determined by indirect fluorescence and the percentage with intact acrosomes as determined by Coomassie brilliant blue staining (B). 0: zero washes; 3: three washes. The data are the means of three separate trials counting 200 cells per treatment per trial. The horizontal lines represent the standard deviations of the means.

Fig. 7. Western blot analysis of sperm extracts, from (F) fresh, (W) washed or (C) capacitated spermatozoa. Fifty micrograms of protein was electrophoresed in each lane. The nitrocellulose was probed with J-23 (J), normal rat serum (R) or with PBS (P). Molecular weight standards: carbonic anhydrase (29 000); lactoglobulin (18 400) and cytochrome c (12 400).

Fig. 8. Indirect immunofluorescent analysis of seminal inhibitor binding to (a) fresh or (b) washed spermatozoa. $\times 750$.

which are both IgM molecules, or PBS for J-23. These treatments did not decrease the percentage of acrosome intact spermatozoa. Furthermore, J-23 induces acrosome reactions in a concentration-dependent manner.

The percentage of acrosome intact spermatozoa in the untreated sample (Fig. 2) is similar to that seen in the PBS and control monoclonal antibody treated groups. This indicates that the procedure used, i.e. incubations and washes, had little effect on acrosome integrity.

The data indicate that the acrosome reaction can be induced in the absence of ZP3, by immunobinding of an epitope on the acceptor. Similar results were recently obtained by immunoaggregation of another putative zona-binding component on murine spermatozoa (Macek *et al.*, 1991). Anti-galactosyltransferase IgG induces acrosomal exocytosis in capacitated cells while the Fab fragment has little effect. Immunobinding of any component in the cap region does not, however, result in the acrosome reaction. C-9 in this study and three components recognized by the murine monoclonal antibodies M5, M41 and M42 (Saling & Lakowski, 1985) have been immunolocalized on the plasma membrane in the cap region of capacitated, living spermatozoa. The ability to localize these antigens suggests that the plasma membrane in the cap region, which is lost with the acrosome reaction, remained intact during the antibody treatments. Thus J-23 is unique, at least among the above antibodies, in its ability to induce the acrosome reaction. Furthermore, J-23 is effective only on capacitated spermatozoa. This requirement was part of the prediction (Saling, 1989) that the acrosome reaction should be induced by immunoaggregation only in capacitated spermatozoa. The observation that J-23 can induce the acrosome reaction in capacitated cells supports the concept that the acceptor may function in zona binding (Poirier *et al.*, 1986).

The number of spermatozoa with equatorial expression of the epitope recognized by J-23 increases with *in vitro* incubation as does the susceptibility to J-23-induced acrosome reactions. However, equatorial expression is not linked to the acrosome reaction since washing induces equatorial expression but does not cause an increase in acrosome-reacted spermatozoa. It is not clear if the washing unmasks equatorial epitopes or if it is the trigger for their redistribution from the cap region. It is not uncommon however for washing to remove loosely bound epididymal components from the sperm head (Saxena *et al.*, 1986). The acrosome reaction is, however, the trigger for the redistribution of galactosyltransferase on murine spermatozoa (Lopez & Shur, 1987) and the PH-20 antigen on guinea-pig spermatozoa (Myles & Primakoff, 1984). Both components

have been designated as secondary binding components which involve interactions between exposed acrosomal components and ZP2, a zona component (Saling, 1989). Galactosyltransferase migrates from the cap region to the equatorial region, whereas PH-20 migrates from the post-acrosomal area to supplement PH-20 on the inner acrosomal membrane.

The acceptor is isolated by affinity chromatography using the seminal inhibitor as the ligand (Aarons *et al.*, 1984). The inhibitor, however, binds only in the acrosomal cap region on both fresh and washed spermatozoa. It has previously been shown that the inhibitor binds in the cap region on spermatozoa capacitated *in vitro* (Boettger *et al.*, 1989) and after incubation for 4 h *in utero* (Robinson *et al.*, 1987). This suggests a functional difference between the cap and equatorial component. This difference is not apparent in the molecular mass of the component recognized by J-23 since a single band at 15 kDa was seen with extract from fresh, washed and capacitated spermatozoa. The fact that both populations of acceptor are equally soluble in the lysis buffer and thus have a chance of being detected on the blot is suggested by the lack of acceptor specific fluorescence seen after the detergent treatment.

The functional significance for the differential inhibitor binding ability is not immediately obvious. However, the presence of a zona-binding site in the equatorial region may have some meaning as suggested in a recent summary of the mechanics of sperm–zona interactions (Baltz *et al.*, 1988). These authors suggested that sperm binding and zona penetration result from a highly organized and regulated series of events involving both tight and loose adhesions and flagellar activity. The tip of the sperm head makes initial zona contact. In the mouse, this is the acrosomal cap region (Bleil & Wassarman, 1983). Subsequently, the spermatozoa lie flat on the zona. This orientation results from the motion of the spermatozoa and binding between the zona and more posterior sites, e.g. the equatorial region. With the acrosome reaction, acrosomal cap components would be eliminated but the equatorial region would remain intact (Yanagimachi, 1981; Saling *et al.*, 1985; Lopez & Shur, 1987). These equatorial sites thus act as a hinge and with flagellar movement the sperm head would move from side to side. This motion tends to create a penetration slit in the zona by the action of the acrosomal enzymes. Although no data exist suggesting that the equatorial acceptor epitope participates in zona binding, the scheme (Baltz *et al.*, 1988) suggests a reason for the exposure of equatorially located zona binding sites before the acrosome reaction.

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References

- Aarons, D., Speake, J.L. & Poirier, G.R. (1984) Evidence for a proteinase inhibitor binding component associated with murine spermatozoa. *Biology of Reproduction* **31**, 811–817.
- Aarons, D., Boettger-Tong, H., Holt, G. & Poirier, G.R. (1991) Acrosome reaction induced by immuno-aggregation of a proteinase inhibitor bound to the murine sperm head. *Molecular Reproduction and Development* **30**, 258–264.
- Baltz, J.M., Katz, D.F. & Cone, R.A. (1988) Mechanics of sperm–egg interaction at the zona pellucida. *Biophysical Journal* **54**, 643–654.
- Battle, T.A., Boettger, H.L. & Poirier, G.R. (1990) An epididymal component capable of masking a sperm head epitope. *Biology of Reproduction* **42**, Supplement I, 111 (Abstract).
- Benau, D.A. & Storey, B.T. (1987) Characterization of the mouse sperm plasma membrane zona-binding site sensitive to trypsin inhibitors. *Biology of Reproduction* **36**, 282–292.
- Bleil, J.D. & Wassarman, P.M. (1980a) Structure and function of the zona pellucida: identification and characterization of the proteins of the mouse oocyte's zona pellucida. *Developmental Biology* **76**, 185–203.
- Bleil, J.D. & Wassarman, P.M. (1980b) Mammalian sperm–egg interaction: identification of a glycoprotein in mouse egg zonae pellucidae possessing receptor activity for sperm. *Cell* **20**, 873–882.
- Bleil, J.D. & Wassarman, P.M. (1983) Sperm–egg interaction: sequence of events and induction of the acrosome reaction by a zona pellucida glycoprotein. *Developmental Biology* **95**, 317–324.
- Bleil, J.D. & Wassarman, P.M. (1988) Galactose at the nonreducing terminus of O-linked oligosaccharides of the mouse egg zona pellucida glycoprotein ZP3 is essential for the glycoproteins sperm receptor activity. *Proceedings of the National Academy of Sciences USA* **85**, 6778–6782.
- Boettger, H., Richardson, R., Free, D., Rushing, S. & Poirier, G.R. (1989) Effects of *in vitro* incubation on

- a zona binding site found on murine spermatozoa. *Journal of Experimental Zoology* **249**, 90–98.
- Florman, H.M. & Wassarman, P.M.** (1985) O-linked oligosaccharides of mouse egg ZP3 accounts for its sperm receptor activity. *Cell* **41**, 314–324.
- Fritz, H., Trautschold, I. & Werle, E.** (1974) In *Methods of Enzymatic Analysis*, pp. 1064–1080. Ed. H. U. Bergmeyer. Academic Press, New York.
- Goding, J.W.** (1986) *Monoclonal Antibodies: Principles and Practice*. Academic Press, New York.
- Haendle, H., Fritz, H., Trautschold, I. & Werle, E.** (1965) Über einen hormonabhängigen inhibitor für proteolytische enzyme in mammalischen accessorischem geschlechtsdrüsen und im sperma. *Hoppe-Seyler's Zeitschrift für Physiologische Chemie* **343**, 185–188.
- Irwin, M., Nicholson, N., Haywood, J.T. & Poirier, G.R.** (1983) Immunofluorescent localization of a murine seminal vesicle proteinase inhibitor. *Biology of Reproduction* **28**, 1201–1206.
- Laemmli, V.K.** (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T₄. *Nature* **227**, 1201–1206.
- Leyton, L. & Saling, P.** (1989a) Evidence that aggregation of mouse sperm receptors by ZP3 triggers the acrosome reaction. *Journal of Cell Biology* **108**, 2163–2168.
- Leyton, L. & Saling, P.** (1989b) 95 kD sperm proteins bind ZP3 and serve as tyrosine kinase substrate in response to zona binding. *Cell* **57**, 1123–1130.
- Lopez, L.C. & Shur, B.D.** (1987) Redistribution of mouse sperm surface galactosyltransferase after the acrosome reaction. *Journal of Cell Biology* **105**, 1663–1670.
- Macek, M.B., Lopez, L.C. & Shur, B.H.** (1991) Aggregation of β -1,4-galactosyltransferase on mouse serum induces the acrosome reaction. *Developmental Biology* **147**, 440–444.
- Moller, C.C., Bleil, J.D., Kinloch, R.A. & Wassarman, P.M.** (1990) Structural and functional relationships between mouse and hamster zona pellucida glycoproteins. *Developmental Biology* **137**, 276–286.
- Myles, D.G. & Primakoff, P.** (1984) Localized surface antigens of guinea pig sperm migrate to new regions prior to fertilization. *Journal of Cell Biology* **99**, 1634–1641.
- Poirier, G.R. & Jackson, J.** (1981) Isolation and characterization of two proteinase inhibitors from the male reproductive tract of mice. *Gamete Research* **4**, 555–569.
- Poirier, G.R. & Nicholson, N.** (1984) Distribution of a proteinase inhibitor of epididymal origin in the tissues and secretions of the male reproductive tract of mice. *Journal of Experimental Zoology* **230**, 465–471.
- Poirier, G.R., Robinson, R., Richardson, R., Hinds, K. & Clayton, D.** (1986) Evidence for a binding site on the sperm plasma membrane which recognizes the murine zona pellucida. *Gamete Research* **14**, 235–243.
- Richardson, R.T., Buckingham, T., Boettger, H. & Poirier, G.R.** (1987) A monoclonal antibody to a zona pellucida–proteinase inhibitor binding component on murine sperm. *Journal of Reproductive Immunology* **11**, 101–116.
- Robinson, R., Richardson, R., Hinds, K., Clayton, D. & Poirier, G.R.** (1987) Features of a seminal proteinase inhibitor–zona pellucida binding component on murine spermatozoa. *Gamete Research* **16**, 217–228.
- Saling, P.M.** (1981) Involvement of trypsin-like activity in binding mouse spermatozoa to zonae pellucidae. *Proceedings of National Academy of Sciences USA* **78**, 6231–6235.
- Saling, P.M.** (1989) Mammalian sperm interaction with extracellular matrices of the egg. *Oxford Reviews of Reproductive Biology* **11**, 339–388.
- Saling, P.M. & Lakowski, K.A.** (1985) Mouse sperm antigens that participate in fertilization. II. Inhibition of sperm penetration through the zona pellucida using monoclonal antibodies. *Biology of Reproduction* **33**, 527–536.
- Saling, P.M., Irons, G. & Waibel, R.** (1985) Mouse sperm antigens that participate in fertilization. I. Inhibition of sperm fusion with the egg plasma membrane using monoclonal antibodies. *Biology of Reproduction* **33**, 515–526.
- Saxena, N.K., Russel, L.D., Saxena, N. & Peterson, R.N.** (1986) Immunofluorescence antigen localization on boar sperm plasma membranes: monoclonal antibodies reveal apparent new domains and apparent redistribution of surface antigens during sperm maturation and ejaculation. *Anatomical Record* **214**, 238–252.
- Shur, B.D. & Hall, N.G.** (1982) A role for mouse sperm surface galactosyltransferase in sperm binding to the egg zona pellucida. *Journal of Cell Biology* **95**, 574–579.
- Wassarman, P.M.** (1990a) Regulation of mammalian fertilization by zona pellucida glycoproteins. *Journal of Reproduction and Fertility Supplement* **42**, 79–87.
- Wassarman, P.M.** (1990b) Profile of a mammalian sperm receptor. *Development* **108**, 1–17.
- Wassarman, P.M., Bleil, J.D., Florman, H.M., Greve, J.M., Roller, R.J., Salzman, G.S. & Samuels, F.G.** (1985) The mouse egg's receptor for sperm: what is it and how does it work? *Cold Spring Harbor Symposia on Quantitative Biology* **50**, 11–19.
- Yanagimachi, R.** (1981) Mechanisms of fertilization in mammals. In *Fertilization and Embryonic Development In Vitro*, pp. 81–182. Eds L. Mastroianni & J. D. Biggers. Plenum Press, New York.

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