Immunodetection of acrosin during the acrosome reaction of hamster, guinea-pig and human spermatozoa

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Mammalian sperm acrosomes contain a trypsin-like protease called acrosin which causes limited and specific hydrolysis of the extracellular matrix of the mammalian egg, the zona pellucida. Acrosin was localized on hamster, guinea-pig and human sperm using monoclonal and polyclonal antibodies to human acrosin labelled with colloidal gold. This was visualized directly with transmission electron microscopy, and with light and scanning microscopy after silver enhancement of the colloidal gold probe. Four distinct labelling patterns were found during capacitation and the acrosome reaction in hamster and guinea-pig spermatozoa, and three patterns were found in human spermatozoa. In the hamster, acrosin was not detected on the inner acrosomal surface after the completion of the acrosome reaction, thus correlating with the observation that hamster spermatozoa lose the ability to penetrate the zona after the acrosome reaction. With guinea-pig and human spermatozoa, acrosin was still detected after the completion of the acrosome reaction, thus correlating with the observation that acrosome reacted guinea-pig spermatozoa bind to and penetrate the zona pellucida.

Key words: acrosin, antiacrosin antibodies, spermatozoa, acrosome reaction.

INTRODUCTION

Spermatozoa associate with the zona pellucida in a two-step process: weak, non-specific primary attachment, easily disturbed by gentle pipetting, is followed by strong, species-specific binding (Hartmann *et al.*, 1972). Primary attachment occurs in heterologous combinations of gametes (Bedford, 1977). The ligand for the sperm receptor of the zona has been characterized in the mouse as an O-linked oligosaccharide on the ZP3 glycoprotein (Florman *et al.*, 1984; Florman and Wassarman, 1985; Vasquez *et al.*, 1989; Wassarman, 1990). Individual ligand glycopeptides bind to sperm, but are not capable of inducing the acrosome reaction. Crosslinking of the glycopeptides on the sperm surface anti-ZP3 antibodies, causes the acrosome reaction, similarly to intact zona pellucida (Leyton and Saling, 1989). Intact zona pellucida induces the acrosome reaction in the hamster (Cherr *et al.*, 1986; Uto *et al.*, 1988), rabbit (O'Rand and Fisher, 1987), bovine (Florman and First, 1989 a, b) and human (Cross *et al.*, 1988) spermatozoa.

During the acrosome reaction, the proenzyme form of acrosin, proacrosin, is activated (for review see Urch, 1991). Proacrosin binds the zona pellucida at a site separate from the proteolytic active site (Urch & Patel, 1991; Jones, 1991), and this binding may help to maintain the sperm on the zona as the acrosome reaction proceeds (Bleil and Wassarman, 1986, 1988). Acrosin also functions to hydrolyze the zona in a limited and specific manner (Urch *et al.*, 1985), although the zona pellucida may not be totally digested or dissolved by the homologous acrosin (Urch, 1986). Acrosin will also hydrolyze heterologous zona; human acrosin completely digests hamster zona pellucida and this hydrolysis can be inhibited with a monoclonal antibody (ACRO-C2E5) to human acrosin (Elce *et al.*, 1986). Acrosin inhibitors prevent zona hydrolysis, and they also prevent sperm-

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zona interaction (Saling, 1981), either by preventing the acrosome from degranulating (Green, 1978), or by inhibiting secondary sperm attachment to ZP2 (Bleil and Wassarman, 1986, 1988). After the acrosome reaction, the limiting membrane of the sperm head is the inner acrosomal membrane. In the golden hamster, once the acrosome reaction has occurred as a result of long sperm preincubation times, the ability to bind and penetrate the zona is lost (Barros *et al.*, 1973, 1984).

For acrosin to function in fertilization, it must be localized in the correct place for activity. Acrosin has been localized on intact sperm of the ram (Huneau *et al.*, 1984), rabbit (Castellani-Ceresa *et al.*, 1983) and human (Tesarik *et al.*, 1988) in the correct position to bind the zona pellucida after the occurrence of the acrosome reaction. However, unequivocal localization of acrosin during capacitation and the acrosome reaction has not been clearly demonstrated. The present study attempts to clarify the localization of acrosin during capacitation and acrosome reaction.

MATERIALS AND METHODS

Recovery and preparation of spermatozoa

Hamster and guinea-pig spermatozoa. These were obtained from the cauda epididymis of adult males. For transmission electron microscopy, spermatozoa were extruded from the cauda epididymis and placed immediately in the fixative and processed as described later for on grid immunoelectron microscopy. In order to study the kinetics of the acrosome reaction with scanning electron microscopy and light microscopy, hamster spermatozoa were suspended in TAPL-10K culture medium (Yanagimachi, 1982; Barros *et al.*, 1984) and incubated for 0, 3, and 6 hours at 37° C. Guinea-pig spermatozoa were incubated in MCM culture medium (Barros, 1974) and incubated for 0, 1, 4 and 7 hours. At the end of each incubation period the samples were processed according to the inmunolabelling technique using the colloidal gold probe and later the samples were silver enhanced (Barros *et al.*, 1990).

Human spermatozoa. They were obtained from samples in an infertility clinic which were normal according to WHO (1989). The samples were processed as described elsewhere (Barros *et al.*, 1988; Barros, 1989) In brief, the semen was washed twice with BWW (Biggers *et al.*, 1971) by centrifugation. After the last wash, the sperm pellet was covered with fresh culture medium and incubated for two hours at 37° C to allow the swim up of the spermatozoa into the culture medium. At the end of this period, the culture medium containing spermatozoa was centrifuged and the sperm suspended into two aliquots. One aliquot was placed immediately in the fixative and processed as described later for on grid immunoelectron microscopy. The other aliquot was incubated in BWW culture medium for O, 3 and 6 hours. At the end of this incubation period they were processed according to the inmunolabelling technique, using the colloidal gold probe and then the samples were silver enhanced.

Antibodies

Antiacrosin monoclonal antibodies, ACRO C2E5 and ACRO B4F6, used in this study, were obtained as described by Elce *et al.* (1986), purified from ascites by the caprilic acid technique (Russ *et al.*, 1983), extensively dialyzed against PBS and adjusted to a final concentration of 2 mg/ml in PBS before use.

Polyclonal antibodies to human acrosin were prepared as described by Leyton *et al.* (1986) and used at the appropriate working dilution in PBS, containing 1% of bovine serum albumin.

"On-grid" immunoelectron microscopy of spermatozoa

For the immunolabeling technique, hamster and guinea pig spermatozoa were obtained from the epididymis and placed immediately in the fixative. Human ejaculated spermatozoa were washed and then selected by swim up, as described above, and then placed in the fixative. The technique used in this study is a modification from the Janssen Biotech AuroProbe EM booklet and introduced by Berríos (personal communication), and is briefly described here. The sperm suspensions

were fixed in a mixture of 4% p-formaldehyde and 0.1% glutaraldehyde prepared in PBS (150 mM NaCl). The samples were dehydrated in a series of increasing concentration of ethanol, embedded in a low viscosity resin (LR Gold Polysciences, USA) and polymerised at 4° C under UV light. Gold sections were mounted on gold 300 mesh grids. The grids were etched for 20 minutes and then transferred to a washing buffer which consisted of a PBS solution with 1% BSA and 0.1% IGSS gelatin (Amersham). After this treatment, the grids were incubated with the washing buffer containing 5% goat blood serum and transferred to a solution containing the polyclonal antiacrosin antibody or the monoclonal ACRO-C2E5 antiacrosin antibody (Elce *et al.*, 1986) and incubated at 37° C for 3 hours. Grids were washed several times and then incubated for 4 hours in drops containing the second antibody conjugated with 10 or 30 nm gold colloidal particles (Amersham). After the antibody treatment, the grids were postfixed in 2.5% glutaraldehyde and stained with uranyl acetate and lead citrate standard methods. The grids were observed with a Siemmens 1A transmission electron microscope.

Immunolabelling of spermatozoa for light and scanning electron microscopy

For light microscope observations, the spermatozoa were placed on a polylysine coated slide (Mazia *et al.*, 1975) on a two well incubation chamber (Berríos, 1989) and then were fixed with 4% p-formaldehyde in PBS. The slides were washed several times with the washing buffer, blocked and treated as above and then incubated with the monoclonal antiacrosin antibody ACRO C2E5 (Elce et al., 1986). After the incubation, the slides were incubated with goat anti-mouse IgG, conjugated to a 1 nm colloidal gold particle and were then silver enhanced (Leunissen *et al.*, 1989). Some slides were studied with a light microscope and other slides, after the silver enhancing treatment, were dehydrated and sputtered with a gold-palladium target and observed with a Jeol J SM-25 SII scanning electron microscope.

Two types of controls were done: a) spermatozoa incubated only with the second antibody, and b) spermatozoa incubated with the pre-immune serum (polyclonal) or with the anti bovine acrosin monoclonal antibody, ACRO B4F6 (Elce *et al.*, 1986), which does not cross react with human, hamster or guinea pig spermatozoa.

RESULTS

Immunoelectron microscopy

Acrosin was localized with the immune serum or the monoclonal ACRO-C2E5 antibody only in the acrosomal region of human, hamster and guinea-pig spermatozoa (Fig. 1A, B, C). No label was found on other regions of the sperm cell, including the equatorial segment (see insert Fig. 1A). The control antibodies did not label spermatozoa.

Immunolabeling of acrosome as seen with light and scanning electron microscopy

Sperm smears of non permeabilized spermatozoa of the three species studied were treated with the gold probe and then silver enhanced. Some spermatozoa, when observed with the light microscope, displayed a distinctive label that appeared as a black precipitate over the acrosomal surface (Figs. 2B,C; 3B,C; 4B,C). When observed with the scanning electron microscope, the label appeared as a coarse precipitate over the acrosomal surface (Figs. 2F,G; 3F,G; 4E,F). The label corresponded to the reaction between the ACRO-C2E5 monoclonal antibody and the corresponding epitope of acrosin present on the acrosome. Therefore this technique was used to study, on each of the species studied, populations of spermatozoa to evaluate the status of the acrosome and the presence or absence of acrosin during capacitation and acrosome reaction.

Kinetics of acrosin labelling during capacitation and acrosome reaction

Hamster. Non permeabilized hamster spermatozoa incubated *in vitro* for different lengths of time, treated with the gold probe and silver anhanced, could be classified into four different patterns, as evaluated with the

Biol Res 25: 31-40 (1992)

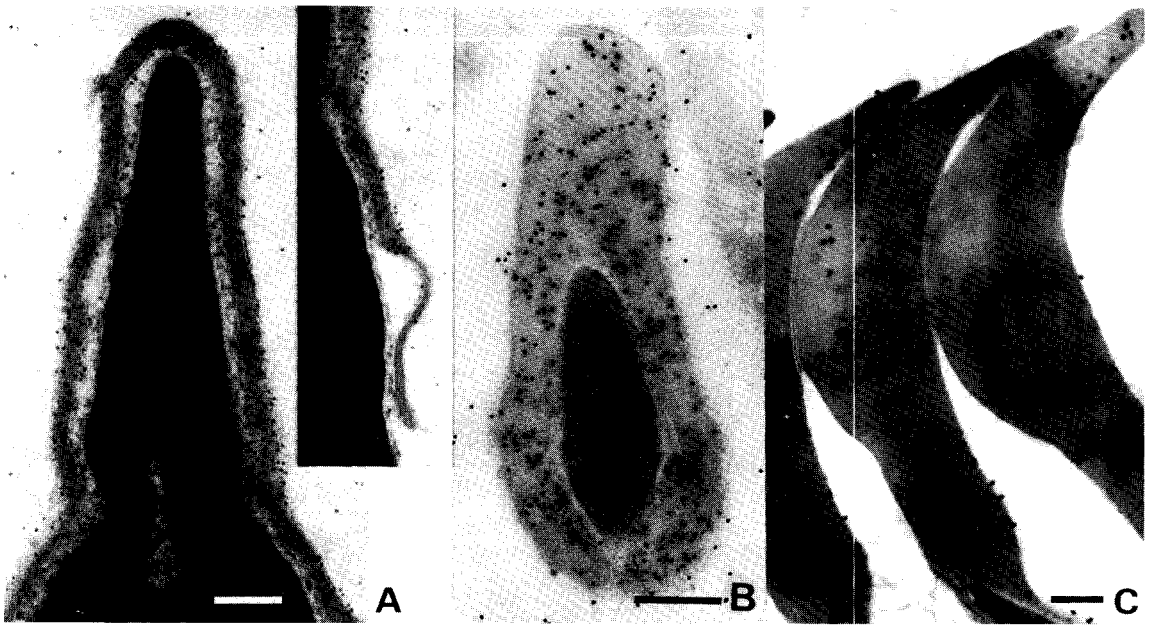

Fig. 1. Immunolocalization of acrosin, using immune serum anti human acrosin, on non reacted spermatozoa of human (A), hamster (B) and guinea pig (C). Note the acrosin labelling associated only in the sperm acrosomal region and not in the equatorial segment (A insert). Bars, 0.2 μm in A and B, 0.4 μm in C.

light or scanning electron microscopes: *pattern 1*, spermatozoa without labelling and an apparently intact acrosome (Figs. 2A,E); *pattern 2*, spermatozoa with intense labelling over the acrosome that remained *in situ* (Figs. 2B,F); *pattern 3*, spermatozoa that showed a reacted acrosome and the label was intense over the inner acrosomal surface and on the acrosomal cap of acrosome reacted spermatozoa (Figs. 2C,G); *pattern 4*, spermatozoa in which the acrosome reaction had been completed and no label was present or only a slight label over the acrosomal collar region (Figs. 2D,H).

The distribution of hamster spermatozoa displaying the different patterns is shown in Table I. At the start of incubation the most abundant pattern was number 1, while at the end of incubation (six hours) the most abundant was number 4 in which no acrosin could be detected on the acrosomal surface.

Guinea-pig. Non permeabilized guinea pig spermatozoa incubated *in vitro* for different times, then treated with the gold probe and silver enhanced, could be classified into four different patterns as evaluated with the light or scanning electron microscopes: *pattern 1*, spermatozoa with no labelling and with an intact acrosome (Figs. 3A,E); *pattern 2*, spermatozoa with the acrosomal cap in place, with an intense label over the main portion of the acrosome and with the acrosomal portion over the flat sides of the nucleus devoid of label (Figs. 3B,F); *pattern 3*, spermatozoa without the acrosomal cap and intense label over the flat surfaces of the nucleus (Figs. 3C,G); *pattern 4*, no label over the acrosomal surfaces of spermatozoa without acrosomal cap (Figs. 3D,H).

TABLE I

Percentage of hamster spermatozoa with diferent patterns of immuno-labelling of acrosin

Sperm Preincubation	PATTERNS OF IMMUNO-LABELLING			
(hours)	1	2	3	4
0	92.3	3.6	0.9	3.4
3	80.3	7.8	0.7	10.2
6	26.4	10.5	5.4	57.9

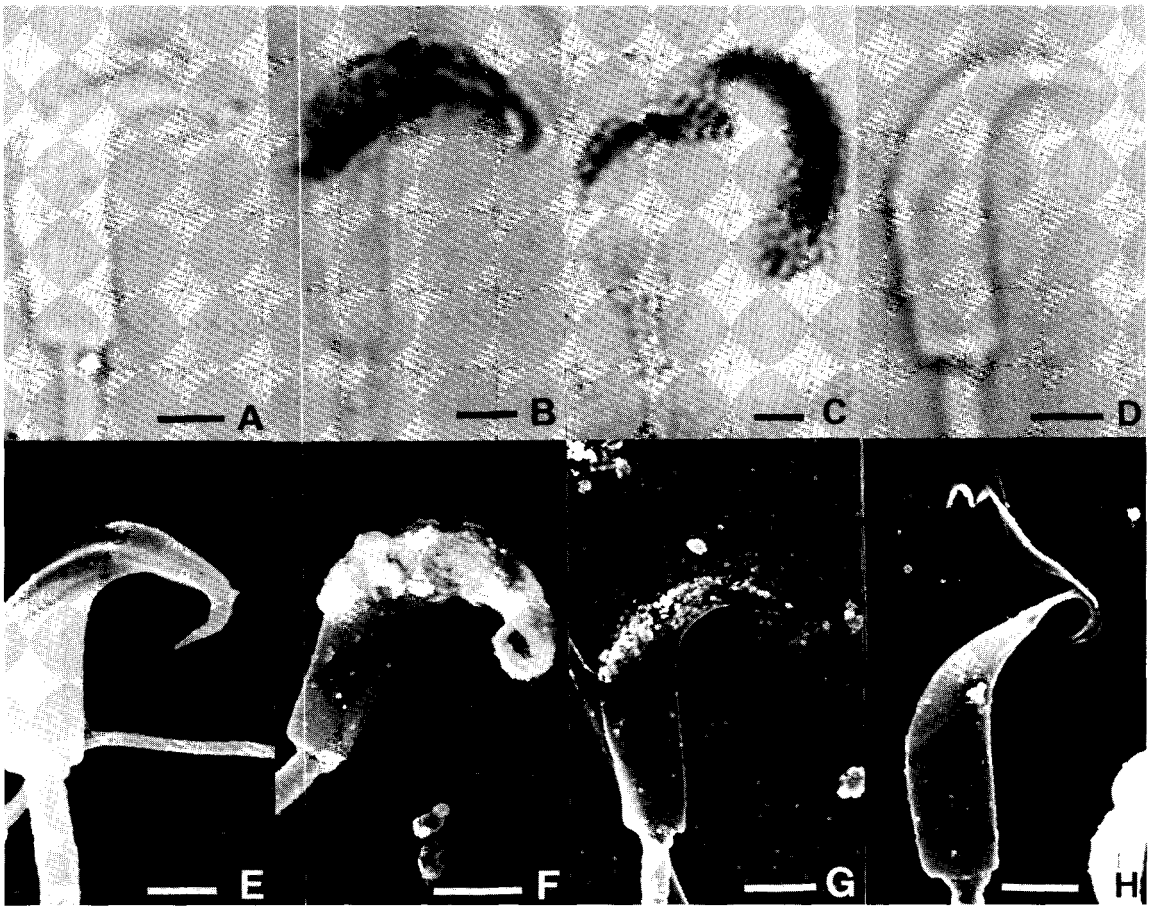

Fig. 2: Immunolocalization of acrosin, with the monoclonal antibody C2E5, on the hamster spermatozoa observed with the phase contrast (A-D) and scanning electron microscopes (E-H). Pattern 1, non reacted sperm without labelling. Pattern 2, intense labelling over an apparently intact acrosome. Pattern 3, reacted spermatozoon with intense labelling over the acrosomal cap and over the inner acrosomal membrane. Pattern 4, reacted spermatozoon without labelling. Bars, 2 μm.

At the start of incubation, pattern 1 was the most abundant (76.6%) and as time of incubation increased the percentage of this pattern decreased (38.6%). Patterns 2 and 3 showed also an increase in percentage as a function of the incubation time. Only small percentage of spermatozoa displaying pattern 4, in which acrosin could not be detected over the inner acrosomal surface, was found throughout the incubation period (Table II).

Human. Non permeabilized human spermatozoa incubated *in vitro* for different times, treated with the gold probe and silver enhanced, could be classified into three different patterns as evaluated with the light or scanning electron microscopes: *pattern 1*, spermatozoa without labelling and an apparently intact acrosome (Figs. 4A,D); *pattern 2*, label over part of rim of the acrosome or over all of it excluding that portion of the acrosome that it

TABLE II

Percentage of guinea pig spermatozoa with diferent patterns of immuno-labelling of acrosin

Sperm Preincubation (hours)	PATTERNS OF IMMUNO-LABELLING 1	2	3	4
0	76.6	11.1	0.7	11.5
1	57.0	22.6	8.6	11.6
4	46.4	28.8	14.2	10.5
7	38.6	37.0	17.3	7.0

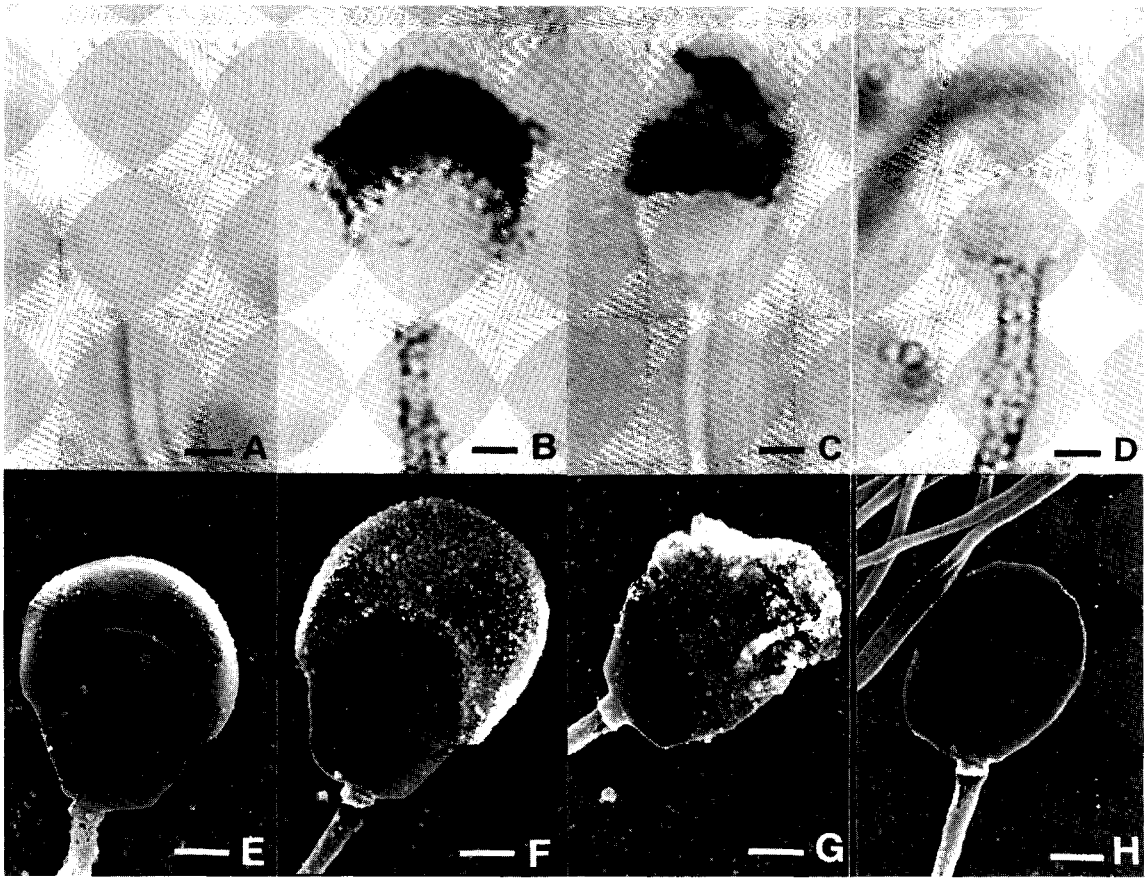

Fig. 3: Immunolocalization of acrosin, with the monoclonal antibody C2E5, on the guinea pig spermatozoa observed with the phase contrast (A-D) and scanning electron microscopes (E-H). Pattern 1, non reacted spermatozoon, without label over the whole spermatozoon. Pattern 2, spermatozoon with intense label over the acrosomal cap only, in which the acrosomal portion over the flat sides of the nucleus was devoid of label. Pattern 3, reacted spermatozoon with intense label over the flat surfaces of the nucleus. Pattern 4, reacted spermatozoon devoid of label over the acrosome. Bars, 2 μm.

is over the flat surfaces of the sperm nucleus (Figs, 4B,E); *pattern 3*, label over the whole acrosome (Figs. 4C,F).

At the start of incubation, most of the spermatozoa (92%) showed no labelling on the acrosomal region and at six hours of incubation this percentage had decreased to 15%. On the other hand, pattern 3 showed an increase from 2.4% to 19% (Table III).

DISCUSSION

Sperm-zona pellucida interaction is a crucial step in fertilization. Weak, non-specific attachment is followed by species-specific binding (Hartmann *et al.*, 1972). The zona pellucida contains ligands for sperm receptors that specifically and tightly bind the spermatozoon to the zona pellucida, and these

TABLE III

Percentage of human spermatozoa with different patterns of immuno-labelling of acrosin

Sperm Preincubation (hours)	PATTERNS OF IMMUNO-LABELLING 1	2	3
0	92.2	5.2	2.4
3	77.6	9.5	12.8
6	15.3	65.7	19.0

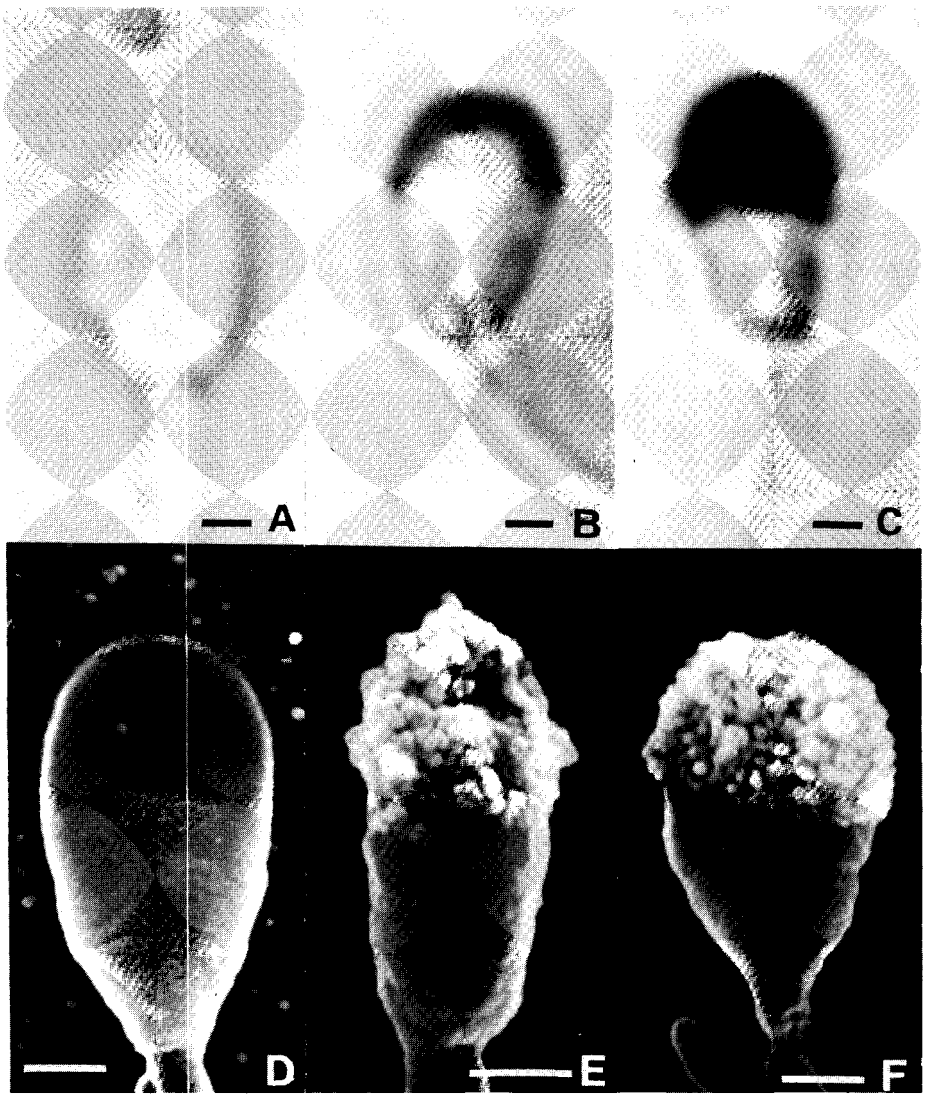

Fig. 4: Immunolocalization of acrosin, with the monoclonal antibody C2E5, on the human spermatozoa observed with the phase contrast (A-D) and scanning electron microscopes (E-H). Pattern 1, spermatozoon without label over the acrosomal region. Pattern 2, spermatozoon labelled over part of the rim of the acrosome excluding the portion of the acrosome that it is over the flat surface of the spermatozoon nucleus. Pattern 3, labelling over the whole acrosome. Bars, 1 μm.

have been characterized in the mouse as O-linked oligosaccharides on the ZP3 glycoprotein (Florman *et al.*, 1984; Florman and Wassarman, 1985, Vasquez *et al.*, 1989; Wassarman, 1990).

Although the follicular and oviducal fluids (Barros, 1968, Barros & Austin, 1967, Yanagimachi, 1969) have the ability to induce the acrosome reaction, the zona pellucida also seems to be able to induce this reaction. This latter idea was based on the findings that hamster acrosomal caps were found attached to the outer edge of the zona pellucida on *in vitro* fertilization experiments. This finding suggested that the acrosome reaction occurred or was completed at the zona pellucida surface (Franklin *et al.*, 1970). This observation was further expanded and evidence was provided for the acrosome reaction inducing properties of mouse zona pellucida (Bleil & Wassarman, 1980, 1983). Similar findings have been reported in hamster (Cherr *et al.*, 1986; Uto *et al.*, 1988), rabbit (O'Rand and Fisher, 1987), bovine (Florman and First, 1989 a, b) and human (Cross *et al.*, 1988) spermatozoa. Multiple connected

ligands of ZP3 appear necessary for the acrosome reaction to occur (Leyton & Saling, 1989). On the other hand it has also been proposed that in the hamster, ZPO added in the oviduct could also be responsible for the induction of the acrosome reaction (Oikawa *et al.*, 1988).

The acrosome reaction results in the physical vesiculation of the outer acrosomal membrane and the overlying plasma membrane (Barros *et al.*, 1967), degranulation of the acrosomal matrix and the release of the acrosomal contents. In this process, proacrosin is activated to acrosin and this conversion is important in both binding of and penetration through the zona pellucida. The loss of zona binding and penetration is correlated with the loss of the acrosome in long incubation time periods in the golden hamster (Barros *et al.*, 1973, 1984).

There are species in which the spermatozoon associates with the zona pellucida through the sperm plasma membrane overlying the acrosome. At the time of fertilization, the spermatozoon detaches from the acrosomal cap and the later is held in place at the zona pellucida surface. Remnants of the acrosomal cap can even remain attached to the zona pellucida after penetration (Franklin *et al.*, 1970; Yanagimachi & Phillips, 1984; Jedlichi & Barros, 1985). As a result of the acrosomal reaction, proacrosin is exposed and becomes activated to acrosin which is thought to assist sperm penetration through the zona pellucida, and acrosin bound to acrosomal membranes may serve to anchor the acrosomal cap on the zona. Moreover, it has been suggested that during acrosome reaction there is a differential release of acrosomal enzymes and acrosin would remain associated to the acrosomal matrix (Hardy *et al.*, 1991).

In the present work we were able to immunolocalize acrosin in golden hamster, guinea pig and human spermatozoa by the use of both polyclonal antihuman acrosin and monoclonal antibovine antibodies. It has been shown that the ACRO C2E5 monoclonal antibody is not recognized by immunoblot, thus suggesting that it recognizes a conformation epitope (Elce *et al.*, 1986). ACRO C2E5 inhibits the digestion of hamster zona pellucida by human acrosin and also inhibits hamster *in vitro* fertilization (De Ioannes *et al.*, 1990) which would suggest that the epitope recognized by ACRO C2E5 is the active site of the enzyme or it is near to it. On the other hand, serine proteases are enzymes that are highly conserved throughout evolution, particularly the active site; thus it would not be surprising that monoclonal antibody ACRO C2E5 cross reacts with hamster, guinea pig and human spermatozoa. Moreover, the immunolocalization with transmission electron microscopy showed that acrosin was located mainly on the main portion of the acrosome of acrosome intact spermatozoa.

The finding that acrosin of hamster supermatozoa is lost after the acrosome reaction and that most of the label remains associated to the acrosomal cap would be consistent with previous findings on the inability to cross the zona pellucida of spermatozoa pre-incubated for long periods of time (Barros *et al.*, 1973, 1984; Capote *et al.*, 1991). However, results obtained with guinea-pig and human spermatozoa are different because there is acrosin still present after the completion of the acrosome reaction. Our finding is consistent with earlier reports showing that acrosome reacted guinea-pig spermatozoa not only bind but also penetrate the guinea-pig zona pellucida (Huang *et al.*, 1981; Huang & Yanagimachi, 1985).

It is important to mention that acrosome reacted guinea-pig spermatozoa do not show proteolytic activity, as evaluated by the gelatin film or by the silver proteinate methods (Huang & Yanagimachi, 1985). This apparent disagreement with our results might be explained considering that guinea-pig spermatozoa, preincubated for long periods of time, lose their ability to fertilize guinea-pig eggs (Fleming & Yanagimachi, 1982), which could be due to the exhaustion of acrosin present in the surface of the inner acrosomal membrane, as shown here. This suggestion is further supported by the finding with SDS-PAGE and labelled fucoidan that proacrosin/acrosin are present on the inner acrosomal surface, after the occurrence of the acrosome reaction (Jones & Williams, 1990).

Human spermatozoa seem to behave in a similar way to guinea-pig spermatozoa in the sense that acrosin seems to remain attached to the inner acrosomal surface after the occurrence of the reaction. The percentage of

spermatozoa displaying pattern 3, *i.e.*, with reacted acrosome, fits well with previous reports on relatively low percentages of spontaneous acrosome reaction (Byrd & Wolf, 1986; Holden *et al.*, 1990). On the other hand, the percentage of spermatozoa displaying pattern 2 could represent acrosin leakage without visible membrane vesiculation, as evaluated with the transmission electron microscope (Tesarik *et al.*, 1990).

The role played by acrosin in fertilization has been recently emphasized by the finding that the fertilizing potential of human spermatozoa is correlated with the total acrosin activity in semen sample (Tummon *et al.*, 1991).

In conclusion, we provide evidence about the location and kinetics of acrosin (or proacrosin) release from the inner acrosomal surface. This evidence is not definite but it supports the idea that acrosin might be involved in sperm passage through the zona.

ACKNOWLEDGEMENTS

Grateful acknowledgements are due to Dr. John Elce for providing us with ACRO C2E5 monoclonal antibody and to Dr. U A Urch and Mr. Jaime Melendez for valuable discussions. The photographic work of Mr. P Cortes is also acknowledged. This work was financed by Grants GA PS 91/01 from The Rockefeller Foundation; 749/91, 577/89 and 684/91 from FONDECYT; and CSA 89/040 from CONRAD. One of us (J.A.C.) acknowledges the support of the Research Training Program in Reproductive Biology of the P. Catholic University of Chile.

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