THE MECHANISM OF SPERM PENETRATION IN STARFISH

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REVIEW:

THE MECHANISM OF SPERM PENETRATION IN STARFISH[1)]

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Introduction

Fertilization consists of a sequential interaction between spermatozoa and eggs, from sperm-egg envelope contact to pronuclear fusion. Mature unfertilized eggs (oocytes) are surrounded by egg envelopes, such as the jelly coat and vitelline coat of sea urchins, egg chorion of mollusc, or zona pellucida of mammals. Fertilizing spermatozoa penetrate the egg envelopes prior to entry into eggs. Many important phenomena involved in sperm-egg interaction, such as the sperm acrosome reaction, egg envelope digestion by sperm lysin, sperm-vitelline coat binding, and egg activation by sperm stimulus, have been reported in detail (cf. Metz and Monroy, 1985). For the success of the fertilization, sperm incorporation into the egg cytoplasm is an important step. However, the details of how sperm enter the oocyte and how they induce egg activation are still unclear. Especially, there are few reports about the penetration of sperm through the egg plasma membrane. This may due to the difficulty of observing the process following passage through the egg envelopes. In natural conditions, only one sperm penetrates in a short period. I succeeded in overcoming these difficulties by using starfish as material, the spermatozoa of which protrude a long acrosomal process after the acrosome reaction and overmature oocytes which have completed both meiotic divisions are fertilized by multiple spermatozoa. This review describes the recent work on sperm penetration, especially what happens to the acrosome-reacted sperm after penetration of the egg envelopes overlying the egg plasma membrane.

Outline of the Fertilization Process in Starfish

Many kinds of marine invertebrate sperm, including those of starfish, have a flagellum providing motility to swim up to the surface of the egg. However, the motility produced by the flagellum is not necessary for penetration of the sperm into

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the egg. Tailless sperm can penetrate into and fertilize oocytes (VACQUIER, 1979; KYOZUKA and OSANAI, 1988b). On the egg surface, sperm undergo the acrosome reaction, an indispensable process for the success of fertilization (TAKAHASHI and SUGIYAMA, 1973).

For the study of fertilization, starfish are one of the most suitable materials, because the spermatozoon possesses a long acrosomal process after the acrosome reaction (DAN, 1954; DAN and HAGIWARA, 1967) (Fig. 1). Initially, the time course of sperm incorporation into the oocyte was determined using a video cassette tape recording system with NOMARSKY differential interference or phase-contrast microscope illumination (Table 1). The fertilizing sperm head remained on the jelly coat for 21.1 s on average, about 10 μm from the egg vitelline coat, and then moved towards the egg surface though the jelly coat. After penetration of the jelly coat, the sperm head again remained stationary on the vitelline coat (elevating fertilization membrane) for 18.3 s. Then the sperm head passed through the elevating fertilization membrane and penetrated the fertilization cone, which was formed temporarily as a protrusion of egg cytoplasm beneath the fertilizing sperm. Sperm head penetration was completed 120.2 s after the addition of sperm. The temporal and spatial separation enabled the observation of the interaction between the fertilizing sperm head and the egg surface separately from that interaction

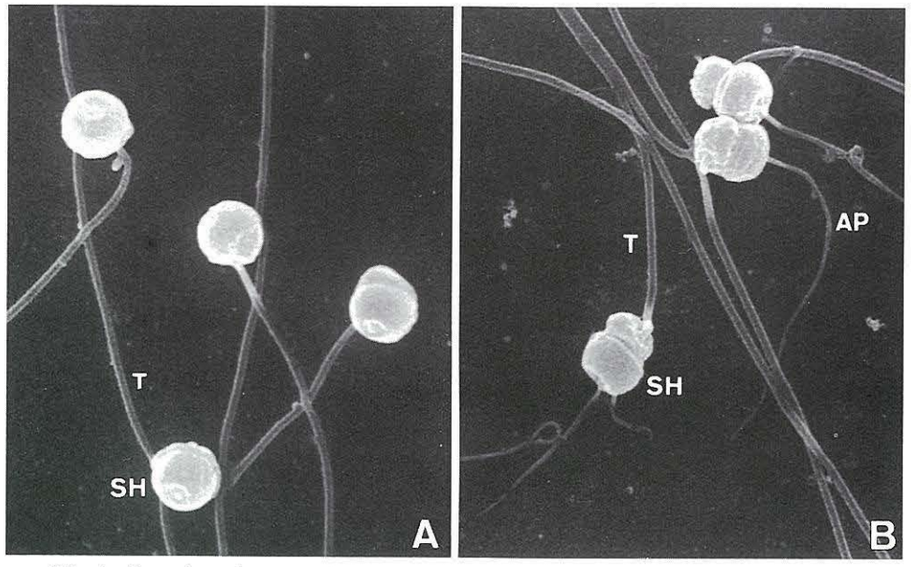

Fig. 1. Scanning electron micrographs of intact and acrosome-reacted sperm of the starfish, *Asterina pectinifera*; intact sperm (A) and acrosome-reacted sperm after treatment with egg jelly solution (B). ×5,480
AP: acrosomal process, T: sperm tail, SH: sperm head

Table 1.
Time course of sperm penetration in starfish, *Asterina pectinifera*

	Average (n=9)	Maximum	Minimum
Arrival of sperm head on the jelly coat surface[1)]	0 (s)	—	—
Beginning of the penetration of the sperm head into the jelly coat[2)]	21.1	37	10
Arrival of the sperm head on the vitelline coat[3)]	77.8	99	48
Beginning of the penetration of the sperm head into the oocyte[4)]	96.1	129	61
Completion of sperm head penetration into the oocyte[5)]	120.2	163	90

Time course of sperm incorporation at each step was determined 9 times using a video recording system. The times measured were the arrival of the fertilizing sperm head on the jelly coat, beginning of sperm head penetration into the jelly coat, attachment of sperm head to the vitelline coat (elevating fertilization membrane), beginning of sperm head penetration into the oocyte through the elevating fertilization membrane, and the completion of sperm head incorporation.

1) Time when the fertilizing sperm head arrived at the surface of the egg jelly coat was determined as zero seconds.
2) Average, maximum, and minimum time when sperm head began to penetrate into the jelly coat after a short resting period on the egg jelly coat.
3) Average, maximum, and minimum time of sperm head arrival at the vitelline coat. The vitelline coat had already detached from the egg plasma membrane and begun to form the fertilization membrane.
4) Average, maximum, and minimum time when the sperm head began to penetrate into the oocyte after a short resting period on the egg surface.
5) Average, maximum, and minimum time when the sperm head penetrated and disappeared from the egg surface.

between the acrosomal process of the sperm and the egg surface. Moreover, over-mature oocytes which have protruded the second polar body become polyspermous readily (FUJIMORI and HIRAI, 1979). Induction of polyspermic fertilization increased the frequency of the opportunity to observe the sperm incorporation process.

There are two main egg envelopes, the jelly coat and the vitelline coat, around the mature oocyte of starfish. The fertilizing spermatozoon penetrates into the oocyte as follows (Fig. 2) (COLWIN and COLWIN, 1965; HIRAI *et al.*, 1981; KYOZUKA and OSANAI, 1988a). (1) Spermatozoa have their own motility due to their flagella. They approached the jelly coat of the egg and underwent the acrosome reaction on its surface. (2) The protruding acrosomal process passed though the jelly coat and vitelline coat and became attached to the egg plasma membrane. (3) A cytoplasmic protrusion developed around the acrosomal process

of the fertilizing sperm. (4) The cytoplasmic protrusion developed into a fertilization cone, the tip of which pointed towards the center of the oocyte. (5) The acrosomal process penetrated at the center of the base (outer surface) of the cone and the sperm head became attached to the base of the cone directly. (6) Cytoplasmic protrusions from the cone trapped the sperm head on the outside of the elevating fertilization membrane (Fig. 3) and incorporated it into the cone (the sperm-engulfing response). (7) The sperm head moved to the inner egg cytoplasm through the fertilization cone (Fig. 4).

In the acrosomal process of the sperm and the fertilization cone, many actin filaments were detected by staining with NBD-phallacidin (Fig. 2). When sperm were added to oocytes in seawater containing cytochalasin B, spermatozoa underwent a normal acrosome reaction and the tip of the acrosomal process attached to the egg plasma membrane. Though egg activation took place, fertilization cone formation and the further sperm-engulfing response did not proceed. The acrosome-reacted spermatozoa remained on the egg surface (KYOZUKA and OSANAI, 1988a). The role of the fertilization cone and the sperm-engulfing response during sperm-incorporation are discussed below.

ROLE OF ACROSOME REACTION IN FERTILIZATION

Spermatozoa undergo the acrosome reaction when they arrive at the egg surface. During the acrosome reaction, polymerization of G-actin took place in the acrosomal process (TILNEY, 1985). Several important events are known to occur during the acrosome reaction (DAN, 1967). These are (1) the release of acrosomal material during the breakdown of the acrosomal vesicle; (2) the exposure of the inner membrane of acrosomal vesicle; and (3) the protrusion of the acrosomal rod or

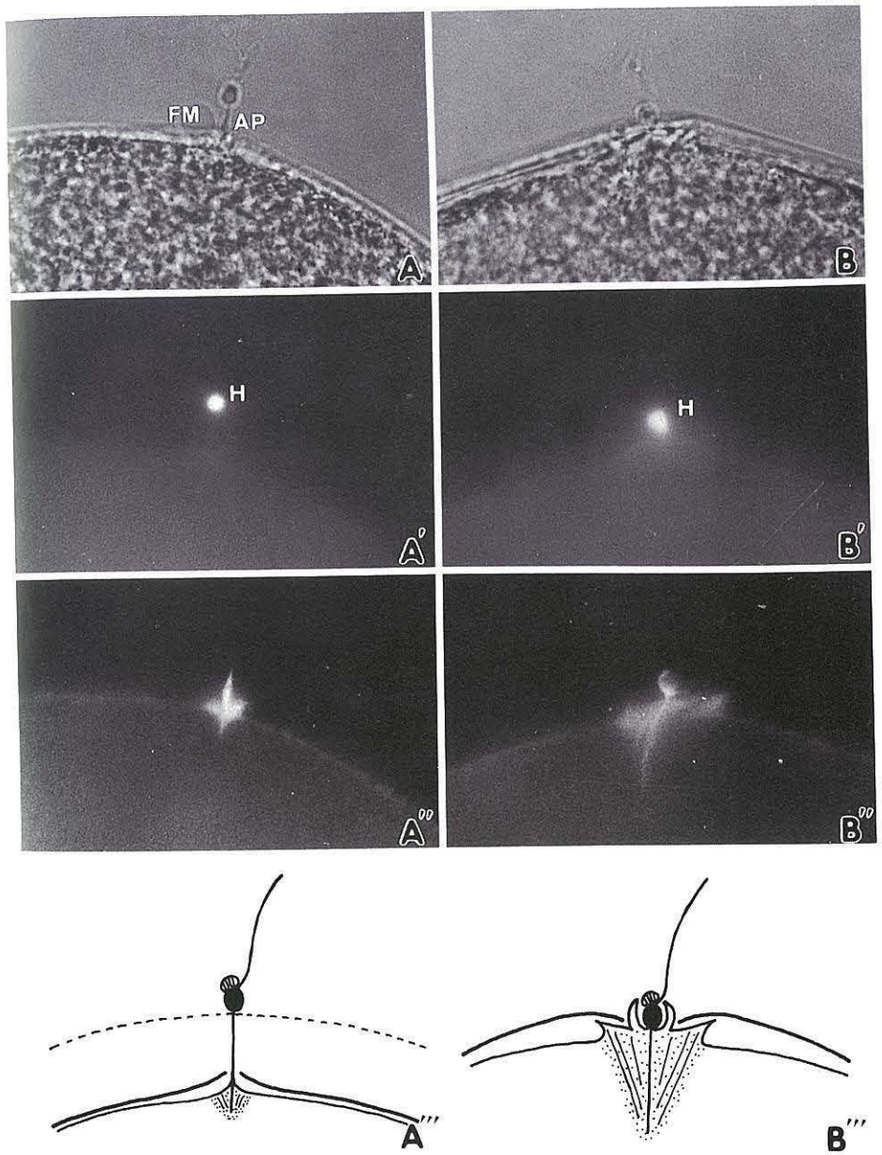

Fig. 2. Sperm incorporation process in the starfish, *Asterina pectinifera*.
Sperm incorporation process in the starfish *Asterina pectinifera* was examined by phase-contrast and epifluorescence microscopy. Sperm were added to mature oocytes and fixed at 1, 1.5, 2, and 3 min with 15% formaldehyde in seawater for 10 min. They were stained with 3.3% NBD (7-nitrobenz-2-oxa-1, 3-diazole)-phallacidin in $Ca^{2+}Mg^{2+}$-free seawater containing 0.1 μg/ml DAPI (4, 6-diamidino-2-phenylindole) for 15 min. ×1,170
(A) 1 min after addition of sperm. Sperm acrosomal process (AP) attached the egg surface and the fertilization membrane (FM) beginning to elevate. (A′) Sperm head (H) detected with DAPI staining was outside the elevating fertilization membrane. (A″) The acrosomal process and the egg cortex beneath the acrosomal process showing strong fluorescence, indicating polymerization of actin filaments. (A‴) Scheme of sperm penetration after addition of sperm.
(B) 1.5 min after the addition of sperm. Phase-contrast microscopy. (B′) Sperm head (H) stained with DAPI has already attached to the egg surface. (B″) Strong fluorescence from the fertilization cone, demonstrating the presence of actin filaments in it. (B‴) Schematic representation of sperm penetration at 1.5 min after addition of sperm.

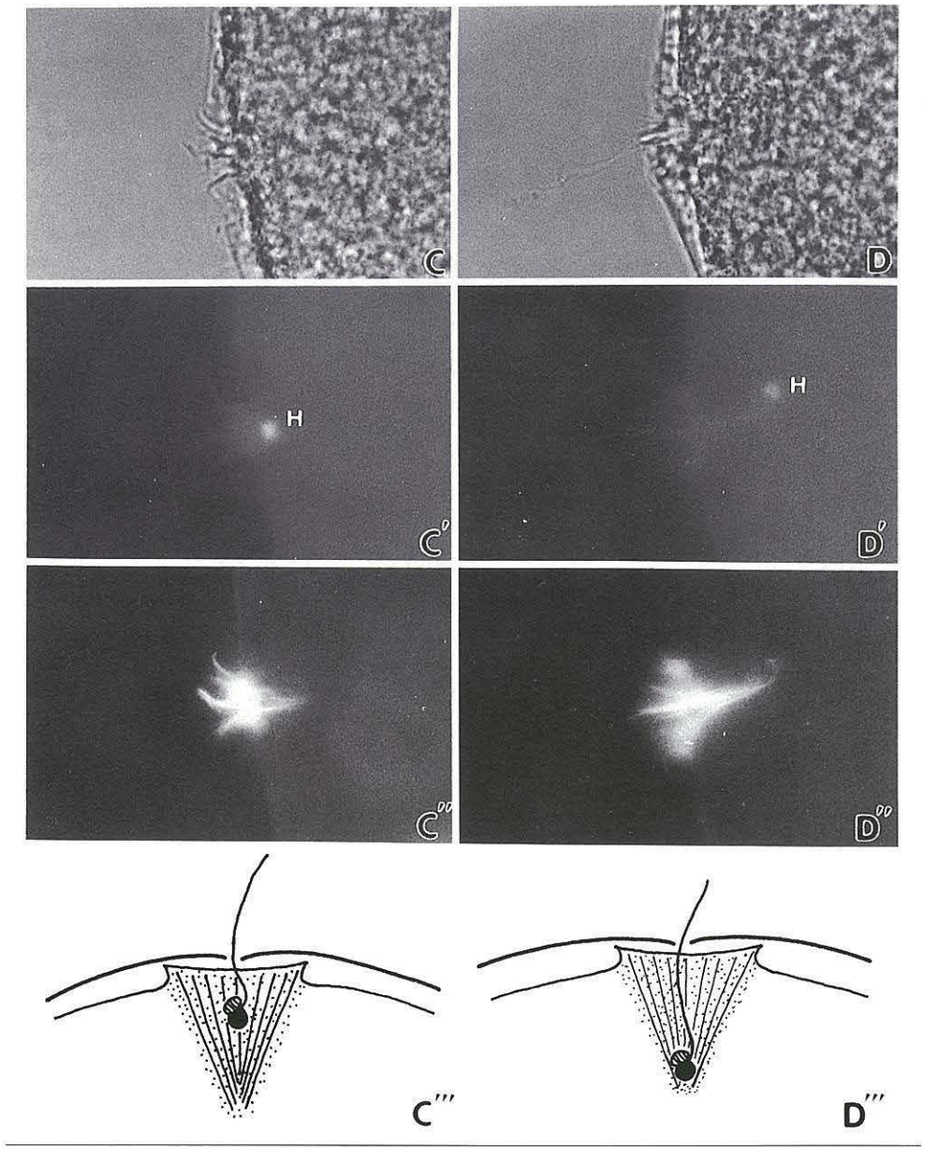

Fig. 2. continue
(C) 2 min after the addition of sperm. (C′) Sperm head (H) has penetrated into the fertilization cone. (C″) Strong fluorescence shows that there are many actin filaments radiating from the center of the oocyte. (C‴) Schematic representation of sperm penetration at 2 min after addition of sperm.
(D) 3 min after the addition of sperm. (D′) Sperm head (H) at the top of a fertilization cone. (D″) Strong fluorescence from the fertilization cone indicating the presence of actin filaments. (D‴) Schematic representation of sperm penetration at 3 min after addition of sperm.

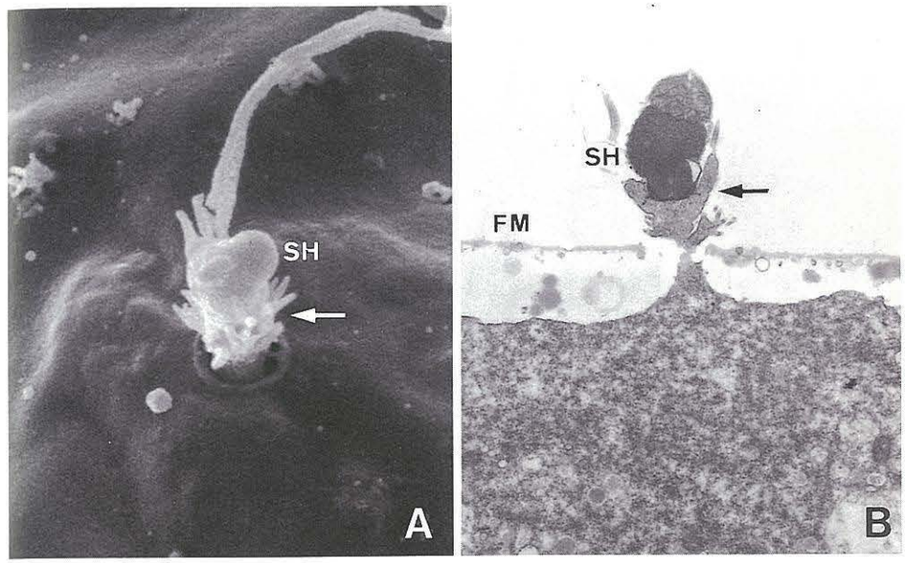

Fig. 3. Electron micrographs of sperm-engulfing response in *Asterias amurensis*. The mature oocytes of *Asterias amurensis* were fertilized, and fixed 2 min after addition of sperm. They were observed by scanning electron microscopy (A) and transmission electron microscopy. ×7,000 The sperm head (SH) is engulfed by the protrusions (arrow) of egg cytoplasm outside the elevating fertilization membrane (FM).

process. There are reports that the acrosomal material plays a part in the digestion of the egg envelopes and species-specific binding between the sperm and the egg surface. Jelly-dispersing substance (Vasseur, 1951; Brookbank, 1958; Isaka *et al.*, 1966) and egg envelope lysin (Harris *et al.*, 1977; Levine *et al.*, 1978) can be detected in sea urchin sperm. These substances act on the jelly coat and vitelline coat of sea urchin eggs respectively and help in the penetration of fertilizing sperm through the egg envelopes. The bindin which is concerned with the species-specific binding of spermatozoa to the vitelline coat is also derived from the acrosomal material (Vacquier and Moy, 1977). Bindin is detected histochemically around the acrosomal rod immediately after induction of the acrosome reaction (Moy and Vacquier, 1979). When acrosome-reacted sperm were treated with trypsin, the electron-dense material around the acrosomal rod disappeared and the spermatozoa lost their ability to fertilize the egg (Sugiyama and Kato, 1977). Bindin of oyster sperm was isolated from the acrosomal vesicle and identified with the electron-dense material at the base of the acrosomal process (Brandriff *et al.*, 1978). The connection between the sperm and the egg chorion mediated by bindin seems to permit the subsequent protrusion of the acrosomal process into the egg chorion (Kyozuka and Osanai, 1985). Christen (1985) reported the isolation of acrosomal

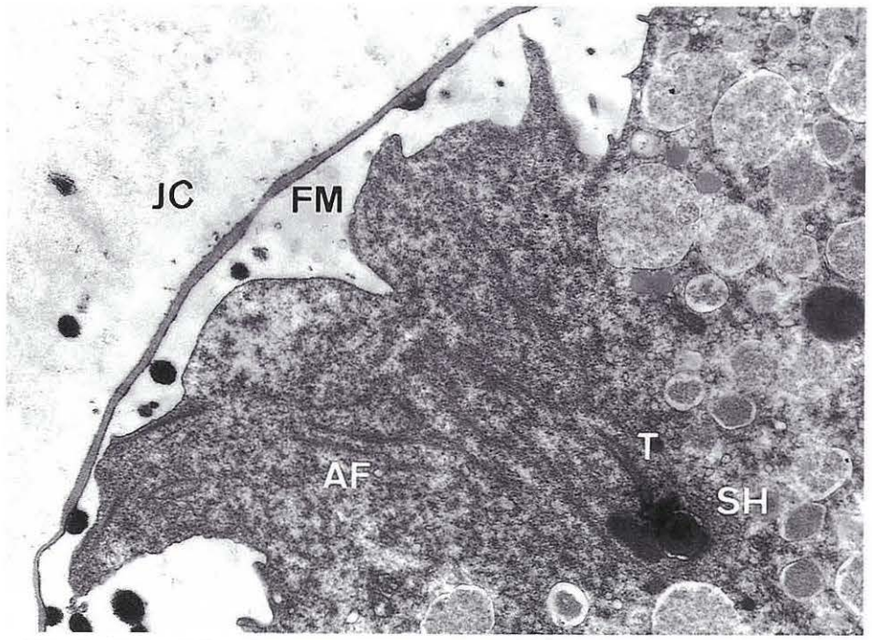

Fig. 4. Transmission electron micrograph of the fertilization cone of the starfish, *Asterias amurensis*.
3 min after the addition of sperm, at a conical process of egg cyoplasm with no yolk or pigment granules, the fertilization cone, has developed. Actin filaments (AF) and the sperm head (SH) can be seen in the fertilization cone. ×7,000 JC : jelly coat on the egg surface, FM : fertilization membrane, T : sperm tail

material from starfish sperm. However, the functions of the acrosomal material in starfish, such as the lysin or species-specific binding activity, are not yet clearly understood.

Egg activation takes place prior to gamete membrane fusion (Hinkley *et al.*, 1986 ; Longo *et al.*, 1986). During fertilization in sea urchins, a rapid change of membrane potential (fertilization potential) occurred (Jaffe, 1976). Bindin prepared from sea urchin sperm induced a change of membrane potential similar to that induced by fertilization by sperm (Longo *et al.*, 1986). The purified bindin induced gamete membrane fusion (Glabe, 1985). Moreover, the acrosomal material from *Urechis* sperm induced cytokinesis in *Urechis* oocytes (Gould *et al.*, 1986 ; Gould and Stephano, 1991). A protease-sensitive factor which reinitiates meiosis was obtained from the acrosomal vesicle of *Mytilus edulis* (Tamaki and Osanai, 1985). These findings show that acrosomal material released during the acrosome reaction of sperm may induce egg activation. In the case of starfish sperm, the breakdown of cortical granules began when the tip of the acrosomal process attached

to, and fused with, the egg plasma membrane (Hirai *et al.*, 1981 ; Kyozuka and Osanai, 1988a). The acrosomal process was cut off immediately after artificial induction of the acrosome reaction, then the acrosomal process-less sperm heads were added to denuded oocytes. These sperm heads were incorporated into the oocyte, but neither egg activation nor gamete membrane fusion took place (Kyozuka and Osanai, 1988b ; 1989). The factor which induces egg activation in starfishes must therefore exist around or on the acrosomal process.

Asterias sperm underwent the acrosome reaction on the jelly coat of the oocyte and protruded the acrosomal process into the jelly coat (Ikadai and Hoshi, 1981). During fertilization in starfish, the tip of the acrosomal process first attached to, and fused with, the egg plasma membrane. Acrosomal process-less sperm heads were incorporated into egg cytoplasm without gamete membrane fusion (Kyozuka and Osanai, 1988b), suggesting that only the acrosomal process possesses the ability to fuse with the egg plasma membrane. The plasma membrane of the acrosomal process is derived from the inner membrane of the acrosomal vesicle (Dan, 1967). Even with spermatozoa of some species in which the acrosomal process is undefined after the acrosome reaction, such as *Hydroides* or mammals, gamete membrane fusion occurred between the newly exposed inner membrane of the acrosomal vesicle and the egg plasma membrane (Colwin and Colwin, 1961 ; Shalgi and Phillips, 1980). In the ordinary fertilization process, gamete membrane fusion must occur at the district parts, the newly exposed inner membrane of the sperm acrosomal vesicle and the egg plasma membrane.

After fusion between the tip of the acrosomal process and the egg plasma membrane, clear egg cytoplasm gathered in the protrusion around the acrosomal process and began to form the fertilization cone. The fertilization cone developed not only as a protrusion of egg cytoplasm along the acrosomal process but also within the egg cytoplasm around the penetrated acrosomal process. In the oyster, clear egg cytoplasm moved along the acrosomal process and formed the penetration cone passing through the thick egg chorion (Kyozuka and Osanai, 1985). On the other hand, spermatozoa of a polychaete, *Tylorrynchus*, did not insert the acrosomal rod into the chorion ; a lobular acrosomal process fused with the tip of egg microvilli containing many actin filaments (Sato and Osanai, 1983). The penetration cone developed along the microvillus fused with a spermatozoon. The actin filaments in the acrosomal process or egg microvilli are necessary for the development of cytoplasmic protrusions such as the fertilization cone or the penetration cone.

Development of the Fertilization Cone and Its Role in Sperm Incorporation

During sperm incorporation, a protrusion of egg cytoplasm (fertilization cone) was formed in which clear cytoplasm containing no yolk or pigment granules

accumulated and actin filaments polymerized within it. When sperm were added to oocytes in seawater containing cytochalasin B, clear cytoplasm appeared around the acrosomal process but actin filaments did not form in it. The fertilization cone did not develop, and the acrosomal process and the sperm head remained on the egg surface. Cytochalasin B blocks sperm penetration in the sea urchins *Urechis* and *Spisula* (GOULD-SOMERO *et al.*, 1977; LONGO, 1978a, b; 1980; BYRD and PERRY, 1980; SCHATTEN and SCHATTEN, 1980, 1981). Though cytochalasin B did not inhibit the acrosome reaction (SANGER and SANGER, 1975), neither the fertilization cone nor the penetration cone developed. As a result, direct contact of the sperm head with the egg surface did not occur.

In the case of mammals, cytochalasin B did not block the penetration of sperm into oocytes (LONGO, 1978b). The acrosomal process does not function in the penetration of egg envelopes. Sperm heads which penetrated egg envelopes attached to the egg plasma membrane by themselves (cf. GWATKIN, 1976; YANAGIMACHI, 1988). Addition of sperm to denuded starfish oocytes in seawater containing cytochalasin B did not inhibit sperm penetration or egg activation (KYOZUKA and OSANAI, in preparation). The movement of egg cytoplasm around the acrosomal process might be necessary for penetration of the sperm head through the egg envelopes and for firm attachment of the sperm head to the egg plasma membrane. The development of the cytoplasmic protrusion with actin filaments is necessary for attachment of the sperm head to the egg plasma membrane prior to the sperm-engulfing response. When oyster sperm were added to isolated egg chorions, they underwent the acrosome reaction and protruded the acrosomal process into the chorion, but the sperm head did not penetrate though the chorion. This shows that the sperm cannot pass though the chorion by itself. The development of the penetration cone from the egg cytoplasm is necessary for the passage of the sperm head through the chorion. The development of this cytoplasmic protrusion must be sensitive to cytochalasin B; however, the incorporation of the sperm head by the engulfing response itself may be insensitive to cytochalasin B.

THE ENGULFING RESPONSE ON THE EGG SURFACE

Penetration of the acrosomal process into the developing fertilization cone led to the attachment of the sperm head to the egg surface. Electron microscopic study showed that the protrusion of egg cytoplasm engulfed the sperm head outside the elevating fertilization membrane. The engulfing response was followed by penetration of the acrosomal process, and was completed within a short period. In normal fertilization, sperm engulfment proceeded simultaneously with gamete membrane fusion and egg activation. Therefore it is very difficult to distinguish these phenomena in sea urchins. As the acrosomal process was long, the fertilization process in starfish could be differentiated into at least two steps, penetration of the

acrosomal process into the developing fertilization cone and the subsequent penetration of the sperm head into the fertilization cone. The engulfing response appeared when the fertilizing sperm head attached to the egg plasma membrane directly. This response of the egg surface was also induced by the sperm head after the acrosomal process had been cut off (KYOZUKA and OSANAI, 1988b). The engulfing response was independent of membrane fusion between the acrosomal process and the egg plasma membrane. Cross-fertilization experiments between acrosome-reacted starfish sperm and denuded sea urchin eggs support this hypothesis. The denuded sea urchin egg surface engulfed the acrosome-reacted sperm head without gamete membrane fusion or egg activation (KYOZUKA and OSANAI, 1988c). This means that direct contact of the acrosome-reacted sperm head with the egg surface is necessary for the sperm-engulfing response. The species specificity of the sperm-engulfing response appears to be weak.

The engulfing response seems to be a kind of phagocytosis. The sea urchin egg surface has the ability to exhibit the phagocytosis of ferritin (CARRON and LONGO, 1984). The denuded egg surface of starfish incorporated the acrosomal process-less sperm head without membrane fusion; however, it did not incorporate acrosome-intact sperm heads or dissected sperm tails (KYOZUKA and OSANAI, 1988b). The selective phagocytotic response against specific substances is incompletely understood. In mouse macrophages, induction of phagocytosis depends on the concentration of certain substances (COHN and PARKS, 1967). Acrosome-intact sperm cannot penetrate the egg envelopes; therefore, they cannot come into contact with the egg plasma membrane (GLABE *et al.*, 1981). Unreacted sperm cannot remain on the egg surface for a long time and soon become separated from it (AKETA *et al.*, 1979). The specific binding between sperm and egg surface is mediated by the acrosomal material, bindin, from the acrosomal vesicle. Tight connection between sperm head and egg surface may be necessary for induction of the sperm-engulfing response.

Polysaccharides activate the phagocytotic response of mouse macrophages (COHN and PARKS, 1967). The acrosomal vesicle of starfish sperm contains polysaccharides (CHRISTIN, 1985). During the acrosome reaction, the electron-dense acrosomal material spreads around the sperm head (DAN and HAGIWARA, 1967). The sperm-engulfing response was induced when the acrosome-reacted sperm head became attached to the egg surface (KYOZUKA and OSANAI, 1988a). The acrosomal material disappeared from the sperm head within a few minutes after the induction of the acrosome reaction. This period corresponded to the ability to induce an engulfing response by the egg surface (KYOZUKA and OSANAI, 1988c). The acrosomal material exposed during the acrosome reaction must be involved in the sperm-engulfing response.

Conclusion

Loeb (1917) thought of fertilization as a kind of phagocytosis. Tyler (1959, 1960) developed his idea, but the theory was not based on experimental results. When a small particle enters a large cell, it is easy to interpret the process as phagocytosis. Colwin and Colwin (1963a, b) first showed clearly, using electron microscopy, that gamete membrane fusion occurred between sperm and egg during fertilization. Hiramoto (1962) showed that a fertilization response was not induced when sperm were introduced into the egg by microinjection. On the other hand, egg or blastomere surfaces had the ability to incorporate ferritin particles or excess sperm (Carron and Longo, 1984; Koehler *et al.*, 1987). Kyozuka and Osanai (1988a) clearly demonstrated the engulfing response toward fertilizing sperm by the oocytes of starfish. They also demonstrated the engulfing response of the denuded sea urchin egg surface to acrosome-reacted starfish sperm heads (Kyozuka and Osanai, 1988c), which was similar to the sperm-engulfing response occurring during the normal fertilization process in sea urchins (Schatten and Mazia, 1976). The positive movement of egg cytoplasm toward the fertilizing sperm was observed in many species (Iwamatu and Ohta, 1978; Elinson, 1978; Colwin and Colwin, 1961; Shalgi and Philips, 1980). The sperm engulfing response must be a universal event through out many species during sperm incorporation into the egg.

Sperm and oocytes are highly differentiated cells essential for the success of fertilization. Eggs become large to accumulate materials necessary for early embryonic development. Egg envelopes consist of complicated structures to ensure the specific binding and selective penetration of only homologous fertilizing sperm, generally a single spermatozoon. Spermatozoa also have many structures corresponding to the egg envelopes, enabling them to bind to and penetrate the egg. There are diverse structures on the egg surface and the spermatozoon to ensure species-specific binding and monospermic fertilization. However, when acrosome-reacted sperm enter the oocyte though the plasma membrane, the sperm-engulfing response, phagocytosis by the egg surface, takes place in many animals. Sperm penetration through the egg plasma membrane must be due to cytoplasmic movement. The sperm penetration process through the egg envelopes may differ as a result of differences in the egg envelopes, the length of the acrosomal process, and the timing of gamete membrane fusion between acrosomal process and egg plasma membrane. However, not only gamete membrane fusion, but also the phagocytotic process (the sperm-engulfing response) of the egg surface seem to be essential for the incorporation of the spermatozoon.

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