Reprod. Nutr. Dévelop., 1986, **26** (4), 999-1008.

Ultrastructural study of the interactions and fusion of ram spermatozoa with zona-free hamster oocytes

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Summary. The interaction of preincubated ram sperm with zona-free hamster oocytes was studied by scanning and transmission electron microscopy. The behaviour of the sperm cells was quite different, depending on whether the acrosomal reaction had taken place or not. The apical ridge of intact spermatozoa contacted the oocyte surface, and egg microvilli spread onto the anterior segment but no fusion ensued. When the acrosomal cap was fenestrated, microvilli were also found on its surface but were then spread over the surface of the postacrosomal region and the equatorial segment of the sperm head lying flat on the egg surface ; fusion with the oocyte occurred in the equatorial segment and extended to the postacrosomal region. Contacts between the microvilli and the inner acrosomal membrane were infrequent and no fusion occurred in the anterior segment. These observations confirm that local changes in the adhesiveness and fusibility of the sperm plasma surface occurred during the acrosome reaction.

Introduction.

As the zona pellucida is the major obstacle to *in vitro* penetration and also the main block to heterospecific fertilization in mammals (Hanada and Chang, 1972), zona-free oocytes have been used for ultrastructural study of the direct interaction of the plasma membranes of the gametes. Particular emphasis was put on the difference in the behaviour of spermatozoa before and after the acrosome reaction. Homospecific interactions have been described by Yanagimachi and Noda (1970a), Phillips and Yanagimachi (1982) and Koehler *et al.* (1984), and heterospecific studies were made essentially with human sperm (Koehler *et al.*, 1984).

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We have undertaken ultrastructural observations of the interactions of zona-free hamster oocytes with ram sperm to try to :
— determine at which stage in relation to the acrosome reaction ram sperm pre-incubated *in vitro* could fuse with zona-free hamster oocytes ; this was a continuation of our work on the factors influencing this gamete interaction (Pavlok and Fléchon, 1985) ;
— correlate changes occurring in the plasma membrane of ram sperm heads during the acrosome reaction (Fléchon, 1985) with the different ways in which the oocyte microvilli interact with the sperm head surface.

Material and methods.

The gametes were collected and incubated *in vitro* as described previously (Pavlok and Fléchon, 1985). In the present experiments, we used only the freshly ejaculated sperm of some of the same rams studied in the preceding paper (Pavlok and Fléchon, 1985) : 2 Caucasian Merino rams, 1 Stavropol Merino ram, 1 Texel × Merino crossbreed ram and 1 ram of undefined crossbreed. The sperm was washed and preincubated for 4 to 6 h in culture medium 199 (Sevac, Praha) supplemented with 34.82 mM sodium bicarbonate, 3.24 mM calcium lactate (B.D.H., Poole), 0.91 mM sodium pyruvate (Serva, Heidelberg), 20 mg/ml of lyophilized calf serum proteins (Sevac, Praha), 50 IU/ml of penecillin (K-salt) and 50 IU/ml of streptomycin sulfate. The pH after equilibration with 5 % CO_2, 5 % O_2 and 90 % N_2 was approximately 7.5. Preincubated sperm (0.2 to 0.8 × 10^{-6} cells/ml) was added to the oocytes in a small volume (150 µl) of culture medium under paraffin oil ; the gametes were incubated for 3 to 19 h.

Part of the material was observed routinely (Pavlok and Fléchon, 1985) by light microscopy in order to control sperm fusion and the formation of the male pronuclei. Batches of eggs from repeated experiments were used for SEM and

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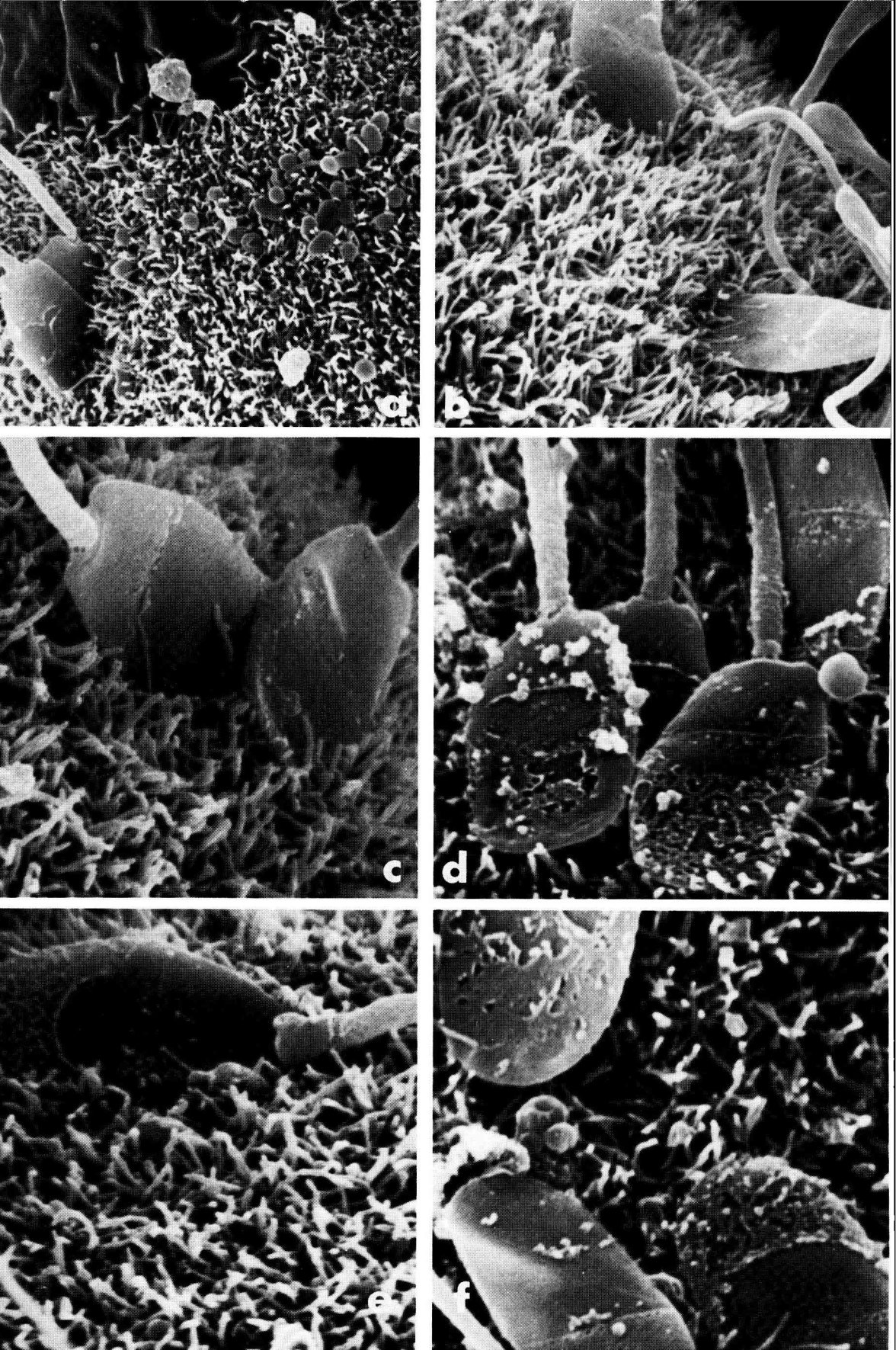
PLATE 1 (SEM)

FIG. a. — *Two spermatozoa on the surface of an oocyte, their apical tip embedded in microvilli.* Note that there are no microvilli on the polar region where there are no spermatozoa ; the globules (≥ 0.2 m) may be extruded cortical granules. Zona-free hamster oocyte (ZFHO) × ram sperm preincubated 6 h ; coculture 4.30 h. × 4,000.
FIG. b. — *At slightly higher magnification the microvilli are seen to adhere to the apical surface of intact spermatozoa.* ZFHO × ram sperm preincubated 5 h ; coculture 4.30 h. × 5,000.
FIG. c. — *Higher magnification shows microvilli spread over the surface of non-reacted sperm heads, except in the postacrosomal region.* ZFHO × ram sperm preincubated 6 h ; coculture 4.30 h. × 9,000.
FIG. d. — *When the acrosome reaction occurs, the microvilli maintain contact with the fenestrated acrosomal cap.* ZFHO × ram sperm preincubated 5 h ; coculture 19 h. × 9,000.
FIG. e. — *Microvilli spread over the postacrosomal region of an acrosome-reacted sperm head with a fenestrated acrosomal cap that is beginning to detach at the limit of the anterior and equatorial segments.* ZFHO × ram sperm preincubated 6 h ; coculture 4.30 h. × 9,000.
FIG. f. — *When the fenestrated acrosomal cap is almost detached, the microvilli are in contact with the postacrosomal and equatorial regions.* There is no clear interaction with the other two heads, one of which has lost its acrosome while the other has not. EFHO × ram sperm preincubated 5 h ; coculture 19 h. × 9,000.


a
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TEM studies. Fixation was carried out with 1 % glutaraldehyde in 0.15 M cacodylate buffer, pH 7.3, at room temperature. For SEM, the material was left in the fixative at 4 °C at least overnight and postfixed in 1 % OsO_4 for 1 h at room temperature. After dehydration in ethanol, it was critical-point dried and coated with gold. For TEM, the specimens were washed in buffer, stored at 4 °C in buffer containing 0.12 M sucrose, postfixed in 1 % OsO_4, stained « en bloc » with uranyl acetate during alcoholic dehydration and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate.

Results.

SEM. — At low magnification, the spermatozoa on the oocyte were randomly dispersed over its surface, except on the polar region (Pl. 1, fig. a). The relationship of the egg to the sperm head depended on the status of the latter, *i.e.* on whether or not the acrosome reaction had occurred. Before the acrosome reaction, only the apex of the sperm head contacted the egg, and egg microvilli were seen adhering on the tip and surface of the acrosomal region (Pl. 1, fig. b). These microvilli sometimes ran a long distance on the sperm head but were generally restricted to the anterior segment (Pl. 1, fig. c).

When the acrosome reaction started (as seen by fenestration of the acrosomal cap), microvilli were still observed on the surface of the anterior segment, and mainly on the apical ridge (Pl. 1, fig. d). The fully-reacted sperm heads (seen by complete fenestration of the anterior segment and detachment of the acrosomal cap from the equatorial segment) were lying flat on the egg surface ; long microvilli then ran over the postacrosomal region and the equatorial segment, but not onto the inner acrosomal membrane of the anterior segment (Pl. 1, figs. e, f).

TEM. — When observed in sagittal, transverse or tangential sections, only the apex of the unreacted sperm heads appeared to contact the egg surface (Pl. 2, figs. a, b, c). The apical ridge was pushed into the egg cortex and the plasma membranes were in close contact in that area (Pl. 2, figs. a, c, d). Egg microvilli adhered to the sperm plasma membrane in the anterior part of the acrosome (Pl. 2, figs. a, b, c), but not in the equatorial segment and post-acrosomal region.

PLATE 2 (TEM)

FIG. a. — *The surface of the anterior segment of a sperm head is in contact with either flat areas of the oolemma or the egg microvilli.* Section tangential to the sperm head. ZFHO × ram sperm preincubated 6 h ; coculture 4.30 h. × 25,000.

FIG. b. — *A consecutive section of the same sperm head showing the egg microvilli spreading over the surface of the anterior segment.* Same material. × 25,000.

FIG. c. — *The apical ridge of the sperm heads is in close contact with the egg surface.* Microvilli adhere to the sperm plasma membrane on the anterior segment. The tangential section of the ooplasm shows many cortical granules. ZFHO × ram sperm preincubated 5 h ; coculture 4.30 h. × 20,000.

FIG. d. — *Detail of the close contact between the plasma membrane* (P) *of the apical ridge* (R) *of a sperm head and the oolemma containing a few endocytotic vesicles* (▶). SFHO × ram sperm preincubated 4 h : coculture 4.30 h. × 23,000.

a
b
c
d

Along the close apposition of the egg surface and the plasma membrane overlying the apical ridge of the sperm head, small pinocytotic vesicles were forming in the oolemma (Pl. 2, fig. d). The adherence of the gamete plasma membranes was so strong that when the acrosomal cap was shed, the plasma membrane of the apical ridge remained on the egg surface.

After the acrosome reaction had started, the microvilli still adhered to the acrosomal cap (Pl. 3, fig. a). Note that fenestration could be inhibited on the side towards the oocyte, perhaps as a result of the close contact with the oolemma. When the acrosomal cap was lost, the microvilli were seldom seen on the inner acrosomal membrane, and cytoplasmic projections of the egg contacted the sperm head in the equatorial and anterior part of the postacrosomal regions (Pl. 3, fig. b). In the next step of gamete fusion, the postacrosomal region was integrated into the egg cortex (Pl. 3, fig. c). There was no fusion with the inner acrosomal membrane, as shown in plate 3 (fig. d). Cortical granules were still visible close to the sperm head of fusing spermatozoa, but the granules were extruded progressively (Pl. 3, figs. c, d). A constant result of heterogametic fusion was the expulsion of the second polar body and the formation of a female pronucleus.

Discussion.

As in an homospecific system or other heterospecific systems (Phillips and Yanagimachi, 1982 ; Koehler *et al.*, 1984), the interaction pattern of zona-free hamster oocytes with mammalian spermatozoa (ram sperm in our experiments) depended entirely on whether the acrosome reaction had occurred or not. It is not known if this reaction can be induced by zona-free eggs (Fraser, 1983), but pictures such as plate 3 (fig. a) suggest that it may occur on the egg surface.

In any case, the interaction of unreacted sperm heads with zona-free oocytes may be regarded as entirely unphysiological, so the meaning of this phenomenon is very speculative, especially as the sperm heads have a tendency to adhere non-specifically to cells or substrates. The binding of the apical plasma membrane to the oolemma appears to be very firm in our heterospecific system and in an

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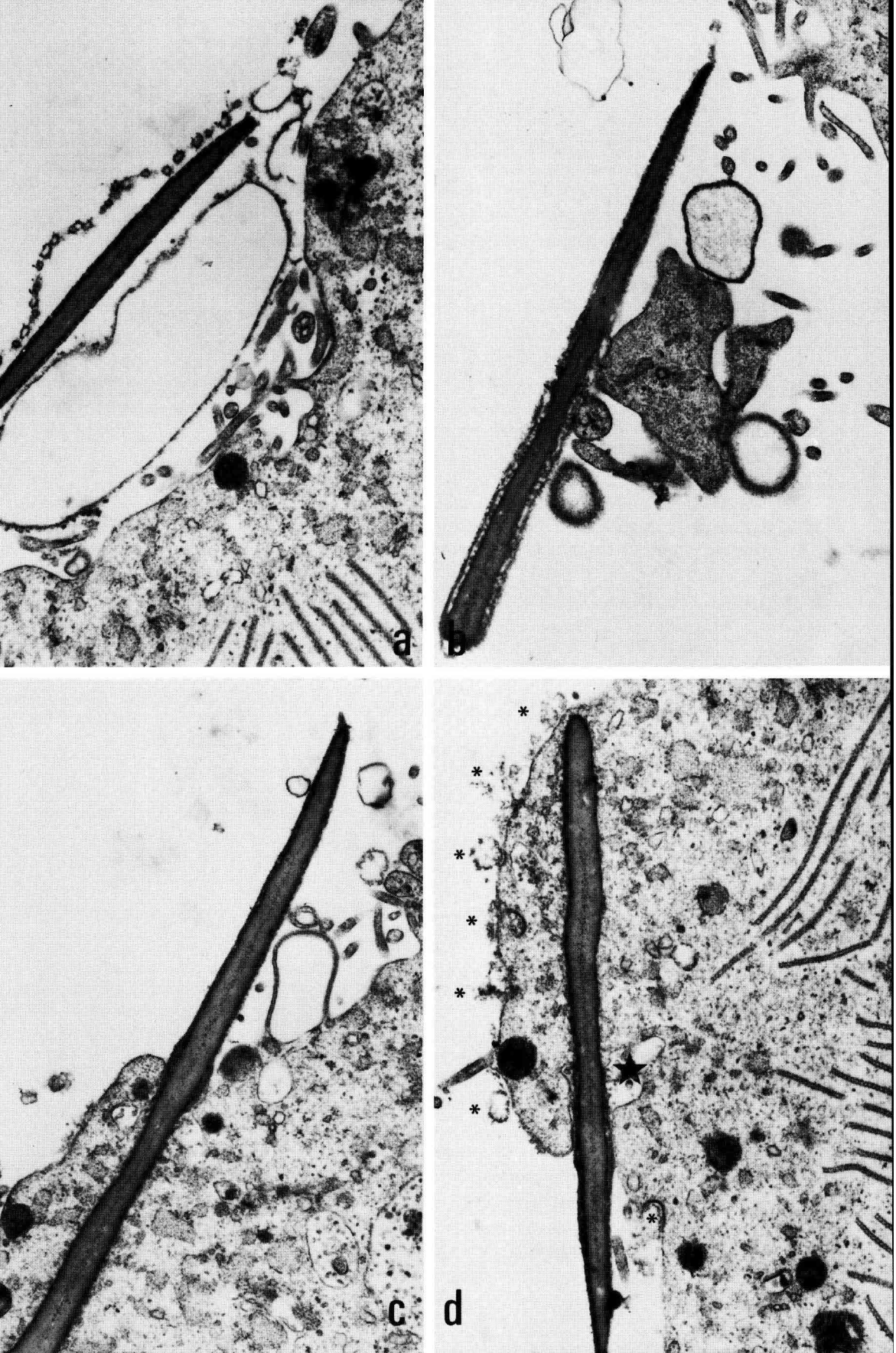
PLATE 3 (TEM)

FIG. a. — *A sperm head undergoing the acrosome reaction on the surface of an oocyte :* there is no fusion between the acrosomal membrane and the plasma membrane in contact with the egg microvilli. ZFHO × ram sperm preincubated 5 h ; coculture 19 h. × 6,500.

FIG. b. — *Acrosomeless sperm head ;* ooplasm projections make contact in the equatorial (and postacrosomal) region. ZFHO × ram sperm preincubated 5 h ; coculture 4.30 h. × 20,000.

FIG. c. — *Sperm head without acrosomal cap in the process of fusion with an oocyte :* the equatorial and postacrosomal regions are already in the ooplasm. No fusion is occurring with the inner acrosomal membrane, although the oolemma forms projections in its vicinity (P). ZFHO × ram sperm preincubated 5 h ; coculture 19 h. × 6,500.

FIG. d. — *Cortical granules are discharged* (*) *close to a sperm head in the process of engulfment of the anterior segment.* The « phagocytotic vesicle » has progressed half way (★). Same material as fig. a. × 6,500.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homospecific one (Yanagimachi *et al.*, 1980). Pinocytotic vesicles found in the oolemma of the contact area are suggestive of the induced endocytosis of some hypothetical receptors. What are the common molecules eventually binding the sperm plasma membrane onto the surface of the zona pellucida and the oolemma ? In the mouse egg, the sperm receptor activity of the zona glycoprotein ZP3 is accounted for by O-linked oligosaccharides which are usually constituents of proteoglycans (Florman and Wassarman, 1985). Proteoglycans are also present in the perivitelline space in at least some mammalian species (Kopečný *et al.*, 1984 ; Talbot, 1985), but their role is unknown.

The morphology of the acrosome reaction observed by SEM corresponds to that described after ionophore treatment (Fléchon, 1985), that is, fenestration of the acrosomal cap starting at the anterior limit of the equatorial segment. The difference in behaviour between acrosome-intact and reacted spermatozoa underlines the changes occurring after the acrosomal reaction has started. First, there is a modification in flagellar activity (Yanagimachi, 1981) which may explain why intact sperm heads first stick perpendicularly onto the oocyte surface, whereas reacted sperm heads are found parallel to the surface. This change of position is more evident in the case of paddle-like sperm heads of boars (Imai *et al.*, 1980), rabbits (Koehler *et al.*, 1984) and rams than in that of pear-shaped human sperm (Koehler *et al.*, 1984) in heterospecific systems, and is intermediary in the case of sickle-shaped hamster sperm in an homospecific system (Phillips and Yanagimachi, 1982). However, this positional effect alone probably cannot explain the change in gamete interactions. As generally observed by the latter authors, egg microvilli contact the equatorial and postacrosomal regions only after the onset of the acrosome reaction. Later on, microvilli are still found on the fenetrasted acrosomal cap but generally not on the inner acrosomal membrane, whereas in human sperm such contacts are not an exception (Koehler *et al.*, 1982 ; Talbot and Chacon, 1982 ; Singer *et al.*, 1985). In homospecific systems, microvillar contacts with the inner acrosomal membrane were observed, but there was no fusion (Phillips and Yanagimachi, 1982 ; Koehler *et al.*, 1984). The important point is that, even if contacts occur with the inner acrosomal membrane, they are transient and no fusion results. This is probably due to the paracrystalline structure and rigidity of the inner acrosomal membrane (Fléchon, 1985 ; Huang and Yanagimachi, 1985).

As in eutherian eggs surrounded by the zona pellucida (Bedford, 1983), the fusion between the microvilli of zona-free eggs and the sperm of acrosome-reacted plasma membrane probably occurs in the equatorial segment or at its border with the postacrosomal region (Yanagimachi, 1981) and extends rapidly to the postacrosomal region in all cases studied, including human sperm (Koehler *et al.*, 1982 ; Talbot and Chacon, 1982). This scheme of initial interaction is confirmed by the apparent limitation of gamete adhesion to the equatorial segment between zona-free eggs and acrosome-reacted sperm of hamster when fusion is inhibited at 10 °C (Hirao and Yanagimachi, 1978 ; fig. 1). It was postulated by Yanagimachi and Noda (1970b) and by Phillips and Yanagimachi (1982) that the acrosome reaction induces a change in the plasma membrane of the equatorial and/or postacrosomal region, allowing fusion. Modifications in the

distribution of sperm surface antigens and intramembranous particles have been effectively observed after induced acrosome reaction (Myles and Primakoff, 1984 ; Fléchon, 1985), which may account for this change.

The spreading of the egg microvilli onto the sperm surface raises the question of their eventual motile role in gamete interaction, as in invertebrates (Schatten and Schatten, 1980). There are arguments in favour of this hypothesis : no contact and fusion occur in the polar region where there are no microvilli ; cortical movements are described after gamete contact in different species (see Yanagimachi and Noda, 1970a) ; microfilaments are present in egg microvilli, and actin is concentrated in the cortex of the fertilization cone in mice (Maro *et al.*, 1984).

Colloque franco-tchécoslovaque,
I.N.R.A., Jouy-en-Josas, 5-6 décembre 1984.

Acknowledgements. — The plates were printed by C. Slagmulder and R. Scandolo and the text edited by Mrs A. Daifuku.

Résumé. *Etude ultrastructurale de l'adhésivité et de la fusion de spermatozoïdes de bélier avec des ovocytes de hamster privés de zone pellucide.*

L'interaction de spermatozoïdes éjaculés de bélier préincubés *in vitro* avec des ovocytes de hamster privés de zone pellucide a été observée en microscopie électronique à balayage et à transmission. Le comportement des spermatozoïdes est très différent suivant qu'ils ont subi la réaction acrosomique ou non. Les spermatozoïdes intacts contactent la surface de l'ovocyte par leur épaississement marginal et les microvillosités de l'ovocyte adhèrent à la surface du segment antérieur, mais la fusion des gamètes ne se produit pas. Lorsque le capuchon acrosomique est fenêtré, il est aussi possible de trouver des microvillosités à sa surface, mais des microvillosités adhèrent alors à la surface de la région post-acrosomique et du segment équatorial ; dans ce cas, la tête du spermatozoïde repose à plat à la surface de l'ovocyte. La fusion avec ce dernier débute au niveau du segment équatorial et s'étend à la région post-acrosomique. Il ne se produit pas d'adhésion ni de fusion entre les microvillosités et la membrane acrosomique interne et le segment antérieur est phagocyté par l'ovocyte. Ces observations confirment que des modifications dans l'adhésivité et la possibilité de fusion avec la surface ovocytaire se produisent localement dans la membrane plasmique du spermatozoïde après la réaction acrosomique.

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