Spreading of a sperm surface antigen within the plasma membrane of the egg after fertilization in the rat

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SUMMARY

The rat sperm surface antigen 2D6, located over the entire surface of the spermatozoon, is shown by use of a monoclonal antibody in indirect immunofluorescence experiments to spread laterally over the surface of the egg after fusion of sperm and egg plasma membranes at fertilization. Freshly fertilized eggs, obtained from superovulated rats 14 h after hCG injection, showed the 2D6 antigen to have spread in a gradient over a discrete fan-shaped area of the egg surface anterior to the protruding sperm tail. Eggs at a later stage of sperm incorporation, obtained 20 h after hCG injection, showed that the spread of antigen had extended to cover most or all of their surfaces. By 40 h after hCG injection, the approximate time that fertilized eggs cleaved to form 2-cell embryos, most of the 2D6 antigen had been lost from the cell surface. Fertilized eggs, but not unfertilized eggs or 2-cell embryos, were lysed by 2D6 monoclonal antibody in the presence of guinea pig complement. A model for sperm–egg fusion is presented to account for the observed pattern of spreading shown by the 2D6 antigen. The possible role of sperm antigens on the egg surface is discussed.

INTRODUCTION

Transmission electron microscopy studies have shown that the process of mammalian fertilization involves fusion of the sperm and egg surface membranes (Szollosi & Ris, 1961; Barros & Franklin, 1968). The sperm organelles thus gain direct access to the ooplasm, whilst the sperm plasma membrane itself remains at the egg surface. The newly formed zygote is therefore bounded by a single plasma membrane which, at least initially, is a composite or mosaic of the two gamete membranes (Austin, 1975). Little is known about the fate of the sperm surface membrane and its components during subsequent development of the zygote.

Using an antiserum to rabbit sperm, O’Rand (1977) was able to detect transfer of sperm surface antigen to the plasma membrane of the rabbit egg at fertilization. Since O’Rand’s detection of antigen was by use of the complement-dependent cytotoxicity test, his findings do not provide any information about

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the distribution of sperm components within the surface membrane of the zygote. An attempt to localize sperm-specific antigens on the fertilized mouse egg by use of indirect immunofluorescence was not successful (Menge & Fleming, 1978). Studies on somatic cells undergoing fusion have shown that there is complete intermixing of the membrane antigens derived from two parental cell types when these fuse to form heterokaryons (Frye & Edidin, 1970). These observations provided some of the earliest evidence that plasma membranes are fluid (Singer & Nicolson, 1972). As indicated by lateral mobility of cell surface components, both spermatozoa (Gaunt, Brown & Jones, 1983) and eggs (Johnson & Edidin, 1978) have plasma membranes which are fluid, although fluidity of the egg membrane decreases after fertilization (Johnson & Edidin, 1978). It might be expected that molecules known to be mobile on the sperm surface will be free to diffuse over the egg surface after fertilization. Surprisingly, Gabel, Eddy & Shapiro (1979a) observed that sperm surface molecules labelled with FITC remained as a discrete patch in both mouse and sea-urchin embryos. This patch persisted throughout subsequent cleavage divisions to be seen on a single blastomere of the 8-cell-stage mouse embryo. It was suggested that the patch might be within the cell surface of the embryo (Gabel et al. 1979a), although subsequent investigation showed that it was internalized (Gundersen, Gabel & Shapiro, 1982).

This paper describes the behaviour of a sperm cell-surface antigen, 2D6, after fertilization in the rat. The antigen, detected by use of a monoclonal antibody, has previously been shown to be mobile in the sperm surface membrane as judged by its susceptibility to antibody-induced patching (Gaunt et al. 1983). It is now shown that the 2D6 antigen is transferred to the plasma membrane of the egg at fertilization, and that it spreads laterally from the site of sperm–egg fusion to cover the entire surface of the 1-cell embryo.

MATERIALS AND METHODS

Collection of eggs, 2-cell embryos, and spermatozoa

Immature female Wistar rats of 28 to 35 days of age were injected intraperitoneally with 10i.u. PMS (Paines and Byrne, Greenford) 48 to 54 h before intraperitoneal injection of 10i.u. hCG (Intervet, Cambridge). For collection of unfertilized eggs, rats were killed at either 14 or 20 h after the hCG injection. The same procedure was followed to obtain fertilized eggs except that females were paired with males after hCG injection. Eggs in cumulus masses were dissected out of the oviducts into PB1/BSA (phosphate-buffered medium containing 4 mg per ml bovine serum albumen, Whittingham & Wales, 1969). 2-cell-stage embryos were obtained from females paired with males and killed 40 h after hCG injection. The embryos were isolated by flushing oviducts with PB1/BSA, using a 33-gauge needle inserted into the open, anterior ends.
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Spermatozoa were collected into PB1/BSA after swimming from cuts made in the cauda epididymidis of mature male rats. Spermatozoa were also collected from the uteri of recently mated female rats.

**Removal of the zona pellucida**

Prior to removal of zonae, cumulus cells were dissociated from eggs by incubation for 5 min at 37°C in PB1/BSA containing 300 units per ml hyaluronidase (Sigma, type IV-S).

Unless otherwise indicated, zonae were removed from eggs and embryos by brief exposure to acid Tyrodes solution, pH 2.5 (Nicolson, Yanagimachi & Yanagimachi, 1975). Zonae were also removed by several other methods. First, they were removed by exposure to oxidizing agents (0.002 M ascorbic acid plus 0.1 volume hydrogen peroxide in phosphate-buffered saline, pH 7.0; method of Braden, 1952). Second, they were removed mechanically by pipetting eggs through fine-bore capillary tubing (Tarkowski, 1961) or by dissection using a micromanipulator (Willadsen, 1979). Third, they were removed enzymically by incubation for 15 min at 37°C in Pronase (Calbiochem, 0.5%; method of Mintz, 1967) or trypsin (Worthington, 0.1%; method of Chang & Hunt, 1956).

**The 2D6 monoclonal antibody**

The 2D6 monoclonal mouse IgM antibody binds to an antigen which is located over the entire surface of rat spermatozoa, which is first acquired during maturation of spermatozoa in the caput epididymidis, and which is susceptible to antibody-induced patching on the sperm surface (Gaunt et al., 1983). Patching of the 2D6 antigen in indirect immunofluorescence experiments is obtained only when 2D6 antibody is diluted below the concentration normally found in hybridoma culture supernatants.

In the experiments now described, undiluted supernatant from 2D6 hybridoma cell cultures was used as the source of monoclonal antibody. The supernatant was HAT medium (Gaunt, 1982) which had supported growth of cells for 12 to 24 h.

**Indirect immunofluorescence**

Labelling was carried out at 4°C in the presence of 0.1% azide. Zona-free eggs, zona-free embryos, or spermatozoa were incubated for 30 min in 1 ml of hybridoma supernatant. They were then washed twice in PB1/BSA, incubated for 30 min in 200 μl biotinylated horse anti-mouse IgG antiserum (1/20 dilution, Vector Labs), washed twice in PB1/BSA, incubated for 30 min in fluorescein-labeled avidin (Sigma), and then given a final three washes in PB1/BSA. Control experiments to assess any non-specific labelling were carried out by incubating eggs in fresh HAT medium, instead of hybridoma supernatant, and then repeating the remainder of the labelling procedure given above. In a small
drop of PB1/BSA on a microscope slide, the labelled eggs were gently com-
pressed by a coverslip which rested on four pillars of silicone grease. The eggs
were then examined using a Zeiss photomicroscope fitted with epifluorescent
illumination and a 40× Neofluar Ph2 objective (N.A. 0.75). Where necessary,
gentle pressure applied to the side of the coverslip allowed the eggs to be rolled
into favourable orientations for microscopy.

Complement-dependent cytotoxicity

Spermatozoa, zona-free eggs, or zona-free embryos were incubated at room
temperature for 30 min in 2D6 hybridoma culture supernatant, washed once in
PB1/BSA, and then incubated at 37 ℃ in 5 % CO₂ atmosphere for up to 2 h in
guinea pig complement (Miles Ltd) diluted one in four in Whittingham’s medium
(Whittingham, 1971). The cytotoxicity tests on eggs and embryos were carried
out in 100 μl drops under paraffin oil (Boots Pharmaceuticals Ltd). Sperm were
judged to have been killed if they became immotile. Eggs were judged to have
lysed if they became ‘ghosts’, showing loss of definition at their margins and
progressive increase in the transparency of their cytoplasm.

RESULTS

Location of the 2D6 antigen on spermatozoa but not on unfertilized eggs

As detected by indirect immunofluorescence using undiluted hybridoma cul-
ture supernatant as first-layer antibody, the 2D6 antigen appeared to be uniformly
distributed over the entire surface of 100 % of spermatozoa taken from the
cauda epididymis of male rats (Fig. 1). The same finding was made for sper-
matozoa extruded from the uteri of recently mated female rats (not shown),
although spermatozoa from this site were tightly agglutinated and were not easily
dispersed for satisfactory labelling and examination. After solubilization and
removal of surface membrane from cauda epididymal spermatozoa, by incuba-
tion for 10 min in 0.1 % Triton X-100, 2D6 antigen was lost from the spermatozoa
as judged from indirect immunofluorescent labelling (not shown).

Fig. 1. Indirect immunofluorescence to show binding of 2D6 monoclonal antibody
to the entire surface of rat cauda epididymal spermatozoa. Left: phase contrast.
Right: u.v. illumination.

Figs 2, 3, 4. Indirect immunofluorescence to detect binding of 2D6 antibody to
fertilized and unfertilized eggs obtained 14 h after hCG injection. In addition to
labelling of the sperm membrane, the egg surface showed patchy labelling
distributed over a fan-shaped area anterior to the protruding sperm tail. This is seen
at the upper surface (Fig. 2) and at the edge (Fig. 3) of the fertilized egg. Unfertilized
eggs, obtained from unmated rats, and also shown in Fig. 2, were not labelled.
Occasionally, up to one-quarter of the sperm tail was incorporated without showing
antigen spread over the egg surface (Fig. 4). Arrows indicate sites of sperm penetra-
tion. Left: phase contrast. Right: u.v. illumination.
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Figs 1–4
The 2D6 antigen could not be detected by indirect immunofluorescence on the surface of unfertilized eggs obtained from superovulated, unmated rats 14 h after HCG (Figs 2, 7), or 20 h after hCG (Fig. 6).

**Localization of 2D6 antigen on the fertilized egg and the 2-cell embryo by indirect immunofluorescence**

Fertilized eggs obtained 14 h after hCG injection (approximately 2 h after ovulation) were usually found to have incorporated one-quarter to one-half of the lengths of their fertilizing spermatozoa (Figs 2, 3, 4). The site of entry of a spermatozoon was often marked by a slight blebbing of the egg surface (arrowed in Figs 2, 4). By indirect immunofluorescent labelling, the 2D6 antigen was seen to be uniformly distributed along the sperm tail protruding from the egg. In addition, patchy labelling was usually seen to extend over the egg surface away from the site of sperm penetration (Figs 2, 3). Of 30 eggs which were judged to have incorporated about one-quarter of the fertilizing spermatozoon, only three failed to demonstrate spread of 2D6 antigen away from the site of sperm penetration (Fig. 4). Eggs which had incorporated more than one-quarter of the fertilizing spermatozoon invariably showed spread of antigen over their surfaces (more than 200 eggs examined). The distribution of 2D6 antigen on the egg surface was not uniform around the site of sperm penetration, but was spread as a gradient over a discrete fan-shaped area anterior to the protruding sperm tail (Fig. 2). When an egg was rolled so that the site of sperm penetration was viewed at its edge, one-quarter to one-third of its margin was seen to be brightly labelled (Fig. 3).

Fertilized eggs obtained 20 h after hCG injection (approximately 8 h after ovulation) were usually found to have incorporated three-quarters or more of the lengths of their fertilizing spermatozoa (Figs 5, 6). The fan-shaped distribution of antigen spreading away from the site of sperm penetration now covered an extensive area of the egg surface (Fig. 5). In some cases antigen had spread over
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Figs 5–7
the entire egg surface to give complete ring fluorescence at the egg margin (Fig. 6).

Two-cell stage embryos obtained 40 h after hCG injection were found to have lost 2D6 antigen from their surfaces. Some of the embryos showed a few patches of weak labelling which were equally distributed over both blastomeres (Fig. 7), whilst others showed no detectable labelling.

To test the possibility that spread of 2D6 antigen over the egg surface might be an artifact associated with the use of acid Tyrode's solution, other methods were employed for the removal of zonae prior to immunofluorescent labelling. Identical results to those described above were obtained after removal of zonae by oxidation using hydrogen peroxide plus ascorbic acid. Spreading of antigen over the egg surface was also seen after zonae had been removed mechanically, although these methods often resulted in loss of the protruding sperm tails. Pronase and trypsin treatments, although effective in zona removal, were unsatisfactory since they also caused loss of 2D6 antigen from both sperm and egg surfaces.

Detection of 2D6 antigen on spermatozoa and fertilized eggs by complement-dependent cytotoxicity

When cauda epididymidal spermatozoa were incubated at 37°C for 30 min in HAT medium, or incubated at 37°C for 30 min in HAT medium followed by 30 min in guinea-pig complement, 50% to 80% of the population were seen to be motile. In contrast, when spermatozoa were incubated at 37°C for 30 min in 2D6 hybridoma culture supernatant followed by 30 min in guinea-pig complement, none of the cells remained motile.

Table 1. Complement-dependent cytotoxicity of 2D6 antibody binding to fertilized eggs

<table>
<thead>
<tr>
<th>Eggs/embryos examined</th>
<th>Treatment prior to incubation in complement</th>
<th>Egg/embryo lysis</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>no. lysed/no. examined</td>
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<tr>
<td>Unfertilized eggs</td>
<td>2D6 antibody</td>
<td>0/50</td>
</tr>
<tr>
<td>Fertilized eggs</td>
<td>2D6 antibody</td>
<td>32/44</td>
</tr>
<tr>
<td>Fertilized eggs</td>
<td>HAT medium</td>
<td>0/44</td>
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<tr>
<td>2-cell embryos</td>
<td>2D6 antibody</td>
<td>0/32</td>
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Experiments were carried out as described in Materials and Methods. Fertilized and unfertilized eggs were obtained 20 h after hCG injection; 2-cell embryos, 40 h after hCG injection. The % lysis obtained in HAT medium followed by complement was taken to indicate background, i.e. lysis which was not due to binding of 2D6 antibody.
Table 1 gives the results of cytotoxicity experiments carried out on unfertilized and fertilized eggs, and also upon 2-cell-stage embryos. Unfertilized eggs were not lysed, whereas fertilized eggs usually were. Of those fertilized eggs which failed to lyse, a few were seen to have the sperm tail projecting from the egg surface, but most appeared to have fully incorporated the spermatozoon. 2-cell-stage embryos were not lysed.

**DISCUSSION**

Examination of fertilized rat eggs by the indirect immunofluorescent labelling technique showed that a sperm surface antigen, 2D6 (Gaunt et al. 1983), spreads laterally from the site of sperm-egg fusion, and that it moves outwards over an extensive area of the surrounding egg membrane. It appeared that the rate of diffusion is slow, so that eggs which had only partially incorporated the fertilizing spermatozoon showed a gradient of antigen which extended over only part of their surfaces. On these eggs the antigen was not uniformly distributed around the site of sperm penetration but was spread over a fan-shaped area anterior to the protruding sperm tail. By the time that eggs had incorporated three-quarters or more of the lengths of their fertilizing spermatozoa, the antigen was spread over most or all of their surfaces. Subsequently, the 2D6 antigen was lost from the surface and only traces of it remained by the time of cleavage to the 2-cell-stage embryo.

The findings now reported do not seem to be consistent with the conclusions of Gabel and coworkers who found that sperm surface molecules labelled with FITC remain as a discrete patch on fertilized mouse and sea-urchin eggs (Gabel et al. 1979a), and that this patch moves to an internal site within the egg (Gundersen et al. 1982). Since the FITC technique is considered to label several different cell surface molecules on spermatozoa (Gabel et al. 1979b), it is surprising that at least some of these do not show the lateral migration over the egg surface now found for the 2D6 antigen. Since the observations made for 2D6 antigen probably refer to a single sperm surface molecule, they do not rule out the possibility that there may be other sperm surface molecules which remain within a discrete patch around the site of sperm penetration. It is possible that the failure to detect 2D6 antigen on the surface of 2-cell embryos may be due to a process of internalization similar to that described by Gundersen et al. (1982). The observations made for 2D6 antigen are in agreement with the earlier work of O’Rand (1977) who found that an antiserum to sperm antigens was cytotoxic to fertilized, but not to unfertilized, rabbit eggs. The present study adds further to O’Rand’s findings in that it demonstrates lateral migration of a sperm antigen to cover the entire surface of the fertilized egg. Yanagimachi, Nicolson, Noda and Fujimoto (1973) observed movement of a membrane receptor in the opposite direction to that now shown for the 2D6 antigen. These authors showed that colloidal iron hydroxide receptors, present in abundance on the unfertilized hamster egg,
migrate on to the postacrosomal surface membrane of the spermatozoon after onset of sperm–egg fusion.

The spread of sperm surface antigen over the egg in a direction mainly anterior to the protruding sperm tail might indicate existence of a unidirectional flow of plasma membrane lipids between two opposite poles of the fertilized egg. It has been suggested that such a flow of membrane lipid in somatic cells might account for the unidirectional movement of surface receptors observed in ‘capping’ experiments (Bretscher, 1976). It seems more likely, however, that the observed distribution of the sperm antigen is a simple consequence of the way in which the rat sperm fuses into the egg. Fig. 8 portrays a model of sperm–egg fusion which is consistent with the observations now described, and which is based upon the model proposed earlier by Szollosi and Ris (1961). After moving obliquely through the zona pellucida (Yanagimachi, 1966; Szollosi & Hunter, 1973), the sperm enters the perivitelline space and comes to lie with its anterior portion over the curvature of the egg. Fusion of the sperm and egg membranes begins in the postacrosomal region (Barros & Franklin, 1968; Yanagimachi & Noda, 1970; Bedford, 1972), so that the contents of the sperm head are the first to enter the egg (Fig. 8A). The site of fusion between the sperm and egg membrane then extends posteriorly along the sperm flagellum, releasing to the egg cytoplasm the contents of the midpiece, the principal piece, and finally the endpiece regions. This process results in a movement of the site of sperm penetration across the egg surface (from X to Y in Fig. 8B). Since, for the egg shown in Fig. 8B, mixing of sperm and egg membranes at X occurs at an earlier time than at Y, a sperm antigen which is mobile in the egg membrane can be expected to have spread laterally from X over a greater distance than from Y. The model thus predicts

Fig. 8. A model for sperm–egg fusion in the rat. The initial site of sperm–egg fusion is at point X (Fig. 8A), but at a later time (Fig. 8B) the site has moved to point Y. Incorporation of sperm membrane components into the egg surface at X therefore begins earlier than at Y. In consequence, lateral diffusion of sperm surface components away from X is more extensive than from Y, and these components spread over a fan-shaped area anterior to the protruding sperm tail.
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a fan-shaped spread of antigen anterior to the protruding sperm tail, which is exactly the distribution now observed for the 2D6 antigen.

It is not known why the 2D6 antigen appeared patchy in its distribution on the egg. One possibility is that patchiness was due to antibody-induced clustering of antigen, but it would not in this case be clear why the labelling conditions used did not also induce patching on spermatozoa. As a second possibility, patchiness of the 2D6 antigen might be a consequence of irregularities such as microvilli on the surface of the egg. It does seem, however, that the egg membrane around the site of sperm penetration is rather smooth in comparison to other regions (Nicosia, Wolf & Mastroianni, 1978; Shalgi, Phillips & Kraicer, 1978). A third possibility is that the sperm membrane, with its associated antigens, does not mix completely into the egg membrane, but remains instead as discrete fragments on the egg surface. It is also unclear why a few eggs failed to show spread of sperm antigen over their surfaces even though they had incorporated up to one-quarter of their fertilizing spermatozoa. Perhaps this finding indicates that there is some initial resistance to the intermixing of sperm and egg membranes.

The observations described in this communication provide no clues as to the function of sperm surface molecules in the membrane of the fertilized egg. Although mammalian eggs may be activated in the absence of spermatozoa by use of several different experimental methods (reviewed by Whittingham, 1980), such parthenogenetic embryos do not develop to term (Whittingham, 1980). The defective development of these embryos is not due to absence of the sperm nucleus (Hoppe & Illmensee, 1982), and so some other sperm component must be essential in the process of normal development. It is possible that this component is a sperm membrane molecule transferred to the egg surface at fertilization. As earlier postulated by Yanagimachi (1977), such a molecule, possibly serving as a channel for the influx or efflux of ions, might be required in the physiological process of egg activation.

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REFERENCES


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