Membrane Flow during Nematode Spermiogenesis

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ABSTRACT Two distinct types of surface membrane rearrangement occur during the differentiation of *Caenorhabditis elegans* spermatids into amoeboid spermatozoa. The first, detected by the behavior of latex beads attached to the surface, is a nondirected, intermittent movement of discrete portions of the membrane. This movement starts when spermatids are stimulated to differentiate and stops when a pseudopod is formed. The second type of movement is a directed, continual flow of membrane components from the tip of the pseudopod to its base. Both membrane glycoproteins and fluorescent phospholipids inserted in the membrane flow backward at the same rate, ~4 μm/min, although their lateral diffusion coefficients in the membrane differ by at least a factor of 5. These observations suggest that pseudopodial membrane movement is due to bulk flow of membrane components away from the tip of the pseudopod.

Membrane components move over the surfaces of many cell types, particularly motile cells. These movements can occur without net membrane rearrangement as typified by the bidirectional movement of surface-attached latex beads on the flagellar membrane of *Chlamydomonas* (6). More often, membrane movement is unidirectional resulting in net rearrangement of membrane components. This is the case for capping of externally cross-linked antigens, lectin receptors and insulin receptors on lymphocytes (23, 24), and for "tipping" of sexual agglutinins on adherent *Chlamydomonas* gametes (11). Amoeboid cells exhibit yet another form of directed membrane movement in which surface markers are transported centripetally from the leading edge toward the cell body (1). Neither the mechanisms underlying these movements nor their physiological significance are understood.

In this study, we have examined membrane movements during differentiation of the amoeboid spermatozoa of the nematode, *Caenorhabditis elegans*. The terminal step in differentiation of these cells is the rapid conversion of spherical, sessile spermatids into polarized, motile spermatozoa. This event can be induced in vitro with the monovalent ion ionophore, monensin (18). It requires extensive cytoplasmic rearrangements: laminar membranes underlying plasma membrane in spermatids accumulate at the base of the pseudopod; membranous organelles (MO) fuse with the plasma membrane; and a pseudopod, which is devoid of organelles but filled with amorphous cytoplasm, extends 3–4 μm from the hemispherical cell body (18, 29).

Here, we demonstrate that extensive surface membrane rearrangement accompanies this cytoplasmic differentiation. Comparison of surface rearrangements during spermiogenesis of wild-type and mutant sperm has revealed two independent types of membrane rearrangement. The first is a nondirected movement of surface markers that occurs only after spermatids are treated with monensin and normally stops when a cell extends its pseudopod. The second is a directed, tip-to-base flow of the entire surface membrane of the pseudopod. In the two following papers it is shown that this pseudopodial membrane flow continues in mature spermatozoa and may propel their amoeboid movement (19, 22).

MATERIALS AND METHODS

Nematode Strains

Worms were grown on *Escherichia coli*-seeded agar petri dish cultures according to Brenner (8). Males of strain CB 1490: *him-5, (el490)* were used as a source of wild-type sperm. Sperm-defective mutant strains included BA524: *fer-1(hc1ts), him-5(el490)*; BA525: *fer-1(hc24ts), him-5(el490)*; BA540: *fer-15(hc15ts), him-5(el490)*; and BA548: *fer-14(hc14), him-5(el490)*. The genetic and phenotypic characterization of *fer-1* is described in references 4, 28, and 29.

Cell-surface Labeling Reagents

Amino-containing latex beads (0.45-μm Diam) were purchased from Polysciences, Inc. (Warrington, Pa.). Rhodamine-labeled wheat germ agglutinin (R-WGA, F/P = 1), a biotin conjugate of soybean agglutinin (Bio-SBA) and ferritin-conjugated avidin (mole ratio, 1) were obtained from Vector Laboratories, Inc. (Burlingame, Calif.). 1-acyl-2-(-4-nitrobenzo-2-oxa-1, 3-diazole)-aminocaproyl phosphatidylcholine (NBD-PC) for preparing fluorescent liposomes was kindly provided by Dr. R. Pagano (Department of Embryology, Carnegie Institution of Washington, Baltimore, Md.), who obtained it from Avanti Biochemical Corp. (Birmingham, Ala.).

Positively Charged Bead Labeling

Males of each nematode strain were isolated as juveniles and maintained as virgins for 2–3 d at 25°C. Nearly all sperm isolated from virgin males are spermatids (18). Cells were isolated by dissecting individual males in a drop of sperm medium (SM, 18) between two vaseline strips on a glass slide. Covering these preparations with a glass slip allowed perfusion of solutions through the ends of the chambers.

To examine the behavior of inert particles on the surface of spermatids during differentiation, cells were treated with SM containing positively charged latex beads (0.25% solids). After 2–5 min, unbound beads were removed by washing with SM. Differentiation of spermatids into spermatozoa was induced by treating the cells with 5×10^{-7} M monensin (18). Movements of beads on differentiating cells were recorded on videotape (Panasonic VTR NV8030) using Nomarski differential interference contrast optics.

Fluorescent Lectin Labeling

Spermatids were treated for 10–15 min with R-WGA (20 μg/ml in SM). The cells were washed and treated with monensin as above. At various times after the start of pseudopod formation live cells were examined and photographed with a Zeiss Universal microscope equipped with epi-illumination for fluorescence. The light source was the 531-nm line from an argon-krypton laser (Control Laser, Inc., Orlando, Fla.) that was defocussed and attenuated for photography. Micrographs were recorded on Kodak Tri-X Pan film developed in Diafine to ASA 1,600.

Biotinylated Lectin/Ferritin-Avidin for Electron Microscopy

To obtain better resolution of the fate of lectin receptors during differentiation we used an indirect labeling technique suitable for transmission electron microscopy (TEM). Spermatids were dissected onto tissue culture plastic. These cells were treated for 10 min with Bio-SBA (100 μg/ml), washed with SM, then incubated for 10 min in ferritin-conjugated avidin (500 μg/ml). Unbound avidin was removed by washing and the spermatids were either fixed immediately in 1.3% glutaraldehyde in 0.95× SM or were activated with monensin and fixed at various times afterward. These preparations were postfixed in OsO_4 and prepared for TEM as described (29). Electron micrographs were taken on a JEOL 100S microscope operated at 80 kV. Prints made at a magnification of 30,000 times were analyzed by determining the number of ferritin grains on 2-cm lengths of the surface membrane (actual length, 0.67 μm). For each cell, all of the periphery that was not sectioned obliquely was scored.

Fluorescent Lipid Labeling

Small lipid vesicles were made by ethanol injection (5, 17) of the fluorescent phospholipid analogue, NBD-PC, into sperm medium containing 10 mg/ml polyvinyl pyrollidone (PVP). Volumes were adjusted to 180 μg lipid/ml of SM, and the preparation of self-quenched, NBD-PC vesicles was dialyzed against SM plus PVP overnight and diluted 1:10 in SM plus PVP for use.

Spermatids were incubated with the NBD-PC vesicles at 2°C for 2 min. Under these conditions the lipid transfers to cell membranes via free monomers not via vesicle fusion (20). The cells were washed with ice-cold SM plus PVP then treated with monensin at room temperature and quickly observed in a light microscope to locate cells forming pseudopods. Differentiating cells were photographed under epifluorescent illumination at various times after the start of pseudopod formation using the 478-nm line of an argon-krypton laser.

RESULTS

Movement of Latex Beads during Spermiogenesis

Spermatids are spherical cells 3–4 μm in diameter. During activation by monensin they rotate intermittently before extending pseudopods (18). To examine surface movements that might account for this rotation we attached latex beads to spermatids and recorded their movements during activation. Fig. 1 shows spermatids and spermatozoa with attached beads, additional examples are shown in reference 22. During activation, latex beads attached to spermatids move independently both during and between periods of cellular rotation. Several examples are shown in Fig. 2. No obvious pattern underlies this movement. On cells with several attached beads, usually one or two beads moved while others remained stationary. Beads on the same cell moved independently, often passing each other as they moved in opposite directions around the edge of the cell or over the dorsal surface. Some cells with one bead moving along the edge had another bead moving simultaneously over the dorsal surface. The movement of each bead was intermittent. While moving, the velocity was usually 10–15 μm/min. Occasionally beads moved over the surface, stopped, and then retraced the same path. More often the paths before and after stopping were different. Cytoplasmic organelles visible under Nomarski optics did not move in concert with beads indicating that movement of surface markers was not correlated with general cytoplasmic flow.

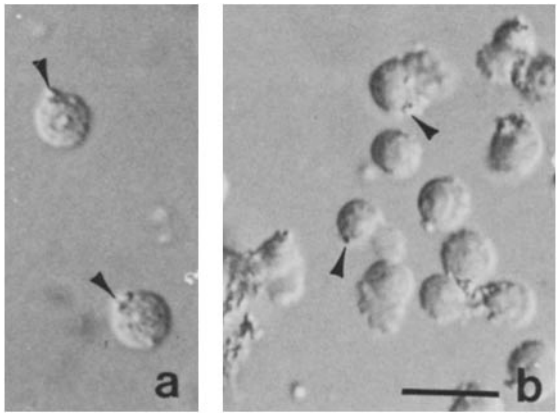

FIGURE 1 Spermatids and spermatozoa with attached latex beads. Nomarski optics: (*a*) Spermatids. (*b*) Spermatozoa. Arrows show beads, the beadlike dots in the center of cells are nuclei. Note that beads are found only on the cell body not the pseudopod of spermatozoa. Bar, 5 μm.

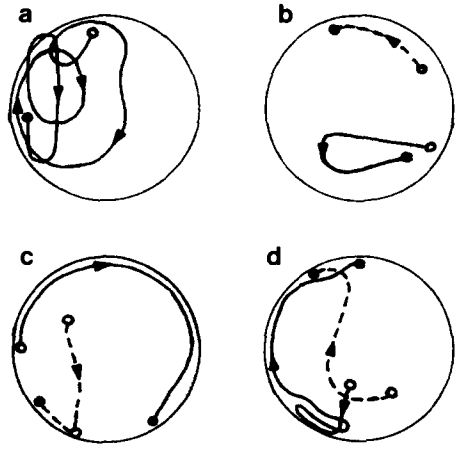

FIGURE 2 Diagrams of the movements of latex beads on wild-type spermatids treated with monensin. Open circles indicate start of track, closed circles indicate end of track, arrowheads show direction of movement. The movements shown are relative to internal organelles to correct for whole cell rotations. (*a*) Path of a single bead moving continuously for 1 min. (*b*) Two beads moving counterclockwise around the periphery of the cell; solid line, bead that moved over the dorsal surface, turned, and returned near to where it started. (*c*) As in *b*, bead marked by the broken line stopped at the second open circle for several seconds then resumed movement around the periphery. Movement of the other bead was continuous. (*d*) Beads that crossed paths twice while moving on the surface.

Both independent bead movement and cellular rotation ceased when activated cells started extending a pseudopod. Beads did not accumulate in any particular pattern but stopped moving anywhere on the hemispherical cell body. However, beads were always excluded from the developing pseudopod.

We compared the behavior of beads on differentiating wild-type cells with that on sperm from three different sperm-defective mutant strains. With the first mutant, defective in gene *fer-15,* only about 0.5% of the spermatids respond to monensin. These few cells become morphologically normal spermatozoa; the behavior of beads on their surface is like that on wild-type. The remaining 99.5% of these mutant sperm do not differentiate, nor do they undergo the rotary movement characteristic of monensin-activated wild-type cells. In keeping with this, we observed no bead movement on their surface although beads attached normally.

We also examined the behavior of beads on two strains with different mutant alleles of gene *fer-1* (*hc1ts* and *hc24ts*). The morphological defects in sperm of both strains are the same: the pseudopods are shorter than normal and MO's do not fuse with the surface membrane (29). Although short, these pseudopods bear pseudopodial projections that undergo the same movements as those on wild-type cells (19). Before pseudopod extension, beads moved on the surface of monensin-activated *fer-1* mutant sperm as on wild-type cells. However, unlike wild-type sperm, bead movement continued on the cell body after these mutant cells extended their short pseudopods. Some of these mobile beads remained on the cell body; others moved onto the pseudopod or were carried out as the pseudopod extended and then were transported back to the cell body (Fig. 3). This was never observed on wild-type cells. We also saw beads moving from side to side on the pseudopod. Thus, although pseudopod extension on *fer-1* mutant sperm creates a morphological polarity, the movement of beads on *fer-1* spermatozoa resembles the wandering bead movements observed on wild-type spermatids before pseudopod extension.

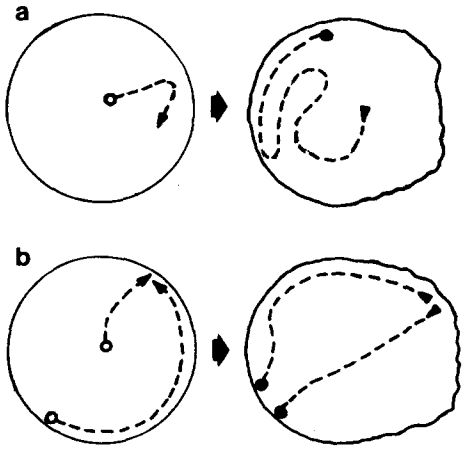

FIGURE 3 Diagrams of the movement of beads on *fer-1* mutant sperm before and after pseudopod extension. Movement was continuous during pseudopod formation. (*a*) Left shows movement on spermatid; bead was in the position indicated by the arrowhead when pseudopod extension began; right shows movement continuing on the cell body after pseudopod formation. The bead did not move onto the pseudopod. (*b*) Two beads moving on the spermatid (left) that were carried onto the extending pseudopod then moved back on the cell body (right).

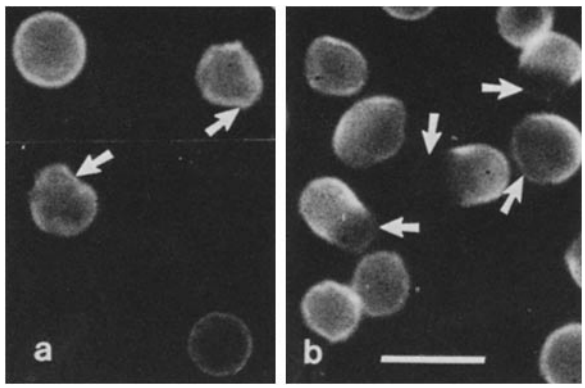

FIGURE 4 Pattern of fluorescence on live sperm treated with R-WGA and activated with monensin. (*a*) Two spermatids showing rings of fluorescence and two spermatozoa with arrow marking the forming pseudopods photographed 15 s after beginning to extend pseudopods. Fluorescence intensity on the pseudopod and cell body are similar. A field of sperm photographed 1 min after pseudopod extension. Arrows mark the pseudopods that are much fainter than the cell bodies. Two spermatids with uniform rings of fluorescence are in the lower left. Bar, 5 μm.

Fate of Surface Lectin Receptors during Spermiogenesis

Argon (3) found that several fluorescently labeled lectins that bind irreversibly to sperm surface glycoproteins stain spermatids symmetrically but stain spermatozoa asymmetrically. He showed that when spermatids labeled symmetrically with lectins are activated to spermatozoa the label becomes confined to the cell body. We have asked how this rearrangement occurs.

When spermatids were labeled with rhodamine-conjugated wheat germ agglutinin (R-WGA) they showed a ring of surface staining with some irregular brighter areas of fluorescence. This staining pattern was stable on each cell. We never detected patching, capping, or other changes in distribution of WGA receptors in spermatids after lectin treatment.

We also observed no change in the fluorescence pattern during the rotation of monensin-activated, R-WGA-treated spermatids. When these cells initially extended pseudopods, the fluoresence intensity of the pseudopod membrane was about the same as that of the cell body (Fig. 4*a*). Therefore, when the pseudopod is formed its membrane must derive, at least in part, from the preexisting surface membrane of the spermatid. Within 30 s after pseudopod formation, however, the fluorescence intensity on the pseudopod was greatly diminished although staining on the cell body was about as bright as that on spermatids (Fig. 4*b* and Fig. 3). We were unable to follow the fate of labeled WGA receptors on individual cells because exciting the fluorophore with laser illumination for more than a few seconds killed the cell. However, we examined many spermatozoa from 30 s to 10 min after the pseudopod formation without finding greater than background fluorescence on pseudopods. This suggests that once labeled lectin receptors were cleared from the pseudopod they did not return.

To examine the movement of a second population of surface lectin receptors in more detail we treated spermatids with biotin-conjugated soybean agglutinin (Bio-SBA) followed by ferritin-avidin. This technique takes advantage of the high affinity binding between avidin and biotin ($K_D = 10^{-15}$ M) and the electron density of ferritin to visualize the location of SBA

receptors with the electron microscope.

Populations of sperm fixed 4 min after monensin treatment included a mixture of spermatids and spermatozoa in the process of extending pseudopods. Activating spermatozoa were recognized by several morphological criteria: few fused MO's, incomplete accumulation of cytoplasmic laminar membranes at the pseudopod-cell-body junction and short pseudopods without projections (18, 29). Electron micrographs of these immature spermatozoa revealed a near uniform distribution of ferritin on the surface of both the cell body and the pseudopod (Fig. 5 *a*; Table I, line 1).

When we fixed labeled cells 6 min after applying monensin we found cells in various stages of differentiation including several full-length pseudopods bearing projections. Because these cells contained many unfused MO's they must be intermediates between immature and fully mature spermatozoa. On some of these cells, the ferritin density was uniform over the surface except at the tips of pseudopodial projections and on membrane adjacent to MO fusion pores (Fig. 5 *b*; Table I, line 2). In these areas the membrane was nearly devoid of ferritin grains. On other cells of this type, ferritin-labeled lectin receptors were absent from the distal portion of the pseudopod but still visible on the pseudopod membrane near its junction with the cell body. On such cells the density of ferritin grains on the membrane at the cell-body-pseudopod junction was greater than on the cell body. The disappearance of ferritin-labeled SBA receptors from the pseudopod was nearly complete on mature spermatozoa obtained by fixing the cells 10 min after monensin treatment. The density of ferritin grains at the base of the pseudopod of the cells was 2–3 times greater than on the cell body (Fig. 5 *c*; Table I, line 3). Many grains appear to pile up away from the membrane. This may be due to cross-linking of the biotin-avidin-ferritin complex.

The rearrangement of SBA receptors during spermiogenesis is qualitatively the same as that observed using R-WGA. Further, the ferritin labeling technique indicates that clearance of lectin receptors from the surface of the pseudopod starts at the tips of the pseudopodial projections and continues centripetally resulting in removal of nearly all lectin receptors from the pseudopod with concommitant accumulation of ferritin at the cell-body-pseudopod junction. We never observed ferritin free in the cytoplasm or bound to any cytoplasmic organelles, even though unstained sections were examined. Thus, the probe was apparently not internalized. The density of ferritin-labeled SBA receptors on the cell body did not change during morphogenesis except that we found that membrane adjacent to MO fusion pores was ferritin-free (Table I, column 1).

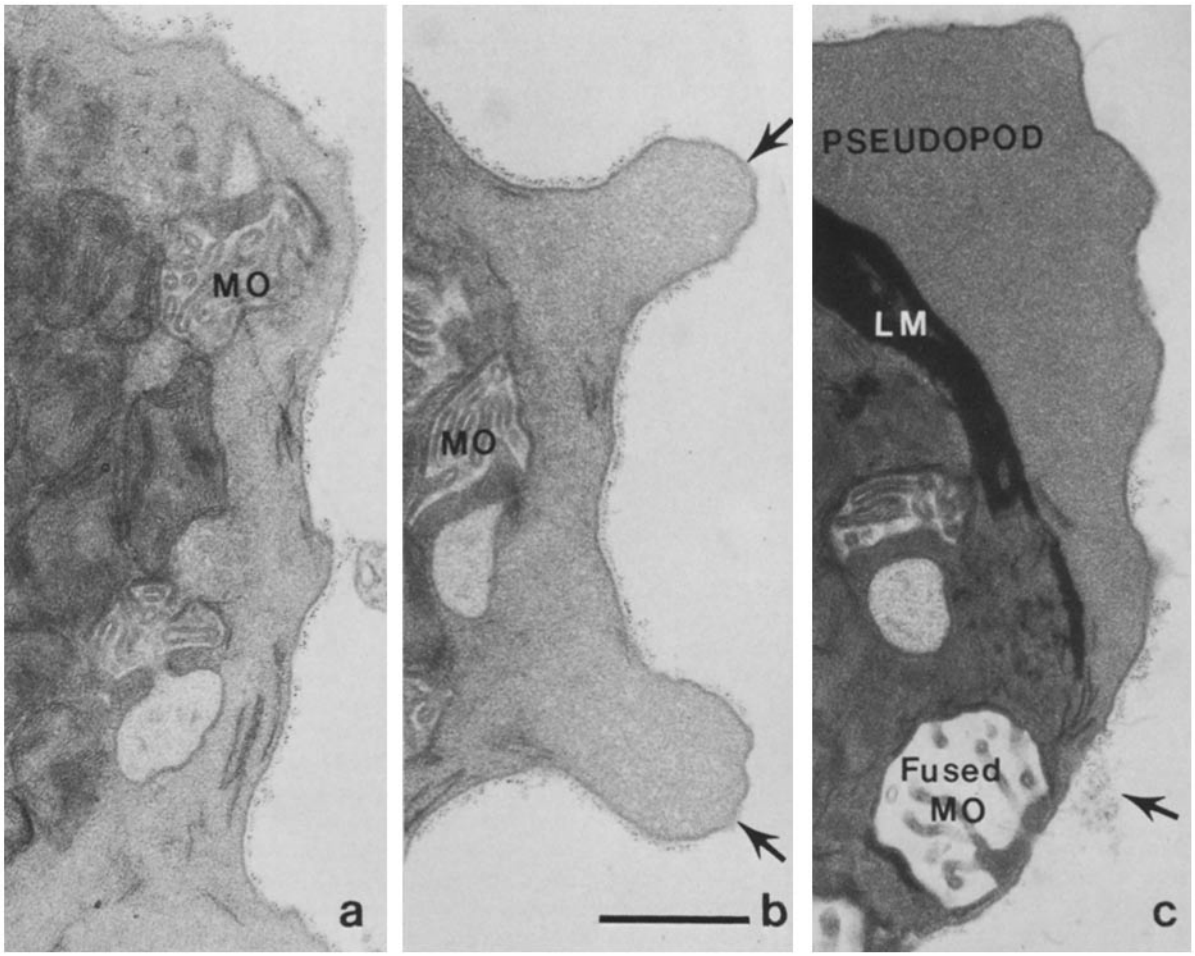

FIGURE 5 Redistribution of SBA receptors. Spermatids labeled with Bio-SBA and ferritin-avidin were activated with monensin and then fixed at various times. (*a*) 4 min, showing the initial stages of pseudopod formation. (*b*) 6 min, the extending pseudopod has formed projections that have become cleared of ferritin (arrows). (*c*) 10 min, a mature spermatozoon showing little ferritin on the pseudopod but a concentration of ferritin at the junction between pseudopod and cell body (arrow). Note that the membrane over the fused membranous organelles (*MO*) is also clear of ferritin. Bar, 0.5 μm. ×39,000.

TABLE I

Ferritin Grain Densities on the Surface of C. elegans Spermatozoa

Class*	Cell body		Pseudopod		Cell body-pseudopod junction‡
	Over fused MO's	Elsewhere	Tips of projections	Elsewhere	
Immature spermatozoa	2 ± 1§	55 ± 13	—	40 ± 16	76 ± 22
Intermediate spermatozoa	1 ± 1	51 ± 17	1 ± 1	48 ± 20	54 ± 15
Mature spermatozoa	3 ± 4	47 ± 10	1 ± 1	6 ± 4	104 ± 34

* Stage of development scored by morphological criteria (see text).

‡ Surface membrane within 1.3 μm on either side of the point where the pseudopod meets the cell body.

§ Entries indicate mean ± SE ferritin grains/0.67-μm segment of surface membrane. Each entry derived from measurement of entire sectioned surface (excluding areas where sections passed obliquely through the membrane) of 2–5 cells.

Redistribution of Membrane-integrated Phospholipid during Differentiation

The movements of both labeled lectins and latex beads report on the behavior of cross-linked surface components. To determine the pattern of movement of a probe inserted directly into sperm membrane, thereby avoiding linkage to external reporters, we treated spermatids with vesicles of the fluorescent phospholipid analogue, NBD-PC. These vesicles are invisible under fluorescent illumination because the fluorophores are so concentrated that they are self-quenched (21, 30). When NBD-PC molecules transfer from the vesicles to the sperm membrane the dilution of the fluorophore in the membrane lipids results in dequenching, a more than two-thousandfold increase in fluorescence, revealing the location of the fluorescent phospholipid in the membrane. Thus when we examined spermatids in solutions of vesicles, we detected a uniform ring of fluorescence around the cells but no background fluorescence.

After washing out vesicle solutions, warming to room temperature and adding monensin, most spermatids extended morphologically normal pseudopods, although some cells lysed. As observed with R-WGA, within the first 20–30 sec after pseudopod extension, the labeled lipid was uniformly distributed around the entire cell including the pseudopod (Fig. 6 *a*). On spermatozoa photographed 30 sec or longer after pseudopod formation the fluorescent probe was barely detectable in the pseudopod membrane (Fig. 6 *b*) but the cell body fluorescence was similar to that on nearby spermatids. These cells also contained several bright fluorescent dots in their cell bodies. Based on their size, number, location, and timing of appearance, these dots are probably fused MO's that label by diffusion of NBD-PC into them in the plane of the membrane.

After several minutes at room temperature much of the label is internalized resulting in a diffuse internal fluorescence along with labeling of the MO's and the nucleus. Thus, except for eventual internalization, both the pattern and timing of rearrangement of NBD-PC on the surface of differentiating sperm was similar to that observed using labeled lectins.

Results of two experiments confirm that NBD-PC was properly integrated into the surface membrane of vesicle-treated cells. In the first, spermatids were treated with either NBD-PC vesicles or R-WGA and activated with monensin. After confirming microscopically that mature spermatozoa had redistributed the fluorescent probes creating the asymmetric pattern described above, the cells were fixed with 1% formaldehyde and 1.2% glutaraldehyde in SM. Fixed, R-WGA-labeled spermatozoa retained their asymmetric staining pattern (not illustrated, staining as in Fig. 4 *b*) indicating that aldehyde treatment stabilized the distribution of lectin-receptor complexes in the surface membrane. In contrast, this fixation did not prevent lateral diffusion of the lipid probe in the plane of the membrane. After fixation, NBD-PC labeled spermatozoa exhibited near-uniform rings of fluorescence indicating return of the probe into the pseudopod surface membrane (not illustrated, pattern as shown in Fig. 6 *a*).

The second experiment compared the effects of the metabolic inhibitor, sodium azide, on the behavior of R-WGA and NBD-PC on mature, asymmetrically labeled spermatozoa. Perfusing 1 mM NaN_3 in SM plus PVP onto mature spermatozoa arrested pseudopod movement and stopped the movement of latex beads attached to the pseudopod (see 22 for a description of bead movement on pseudopods). Treating asymmetrically labeled, R-WGA-stained spermatozoa with 1 mM NaN_3 resulted in return of lectin-receptor complexes onto the pseudopod. The intensity of fluorescence in the pseudopod membrane increased gradually for 70–80 sec after azide treatment (Fig. 7 *a–c*) and then remained stable. The return of fluorescence to the pseudopod membrane of NBD-PC-labeled, azide-treated spermatozoa was complete in nearly all cells within 15 sec after drug perfusion (Fig. 8 *a, b*). Labeling patterns at shorter intervals could not be determined because of the time needed to complete azide perfusion then refocus and photograph the cells. The effects of azide were reversible. Within a few seconds after washing out the drug the spermatozoa began ruffling their pseudopods and by 60 sec these cells cleared their pseudopods of either R-WGA or NBD-PC (Fig. 7 *d*).

DISCUSSION

This study demonstrates two strikingly different patterns of rearrangement of membrane components on differentiating *C. elegans* sperm. The first is nondirected movement of membrane-bound beads on the surface. It occurs after spermatids are activated with monensin but ceases when the cell extends a pseudopod. The second is the directed clearance of lectin receptors and integrated fluorescent phospholipid from the surface of the mature pseudopod.

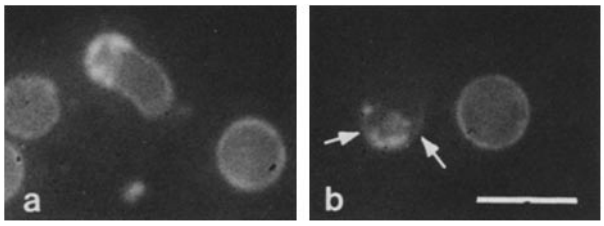

FIGURE 6 Pattern of fluorescence on live sperm incubated in NBD-PC vesicles. (*a*) Round cells are spermatids exhibiting a uniform ring of fluorescence and no staining of cytoplasmic organelles; spermatozoon photographed 15 sec after pseudopod extension. Fluorescence intensity of pseudopod membrane is about the same as that on nearby spermatids. Bright dots in the cell body are fused membranous organelles. (*b*) Spermatid (right) and spermatozoon (left) photographed 1 min after extending its pseudopod. Intensity of fluorescence in cell body membrane (arrows), visible among the membranous organelles is about the same as on the spermatid membrane but fluorescence is barely detectable in the pseudopod. Bar, 5 μm.

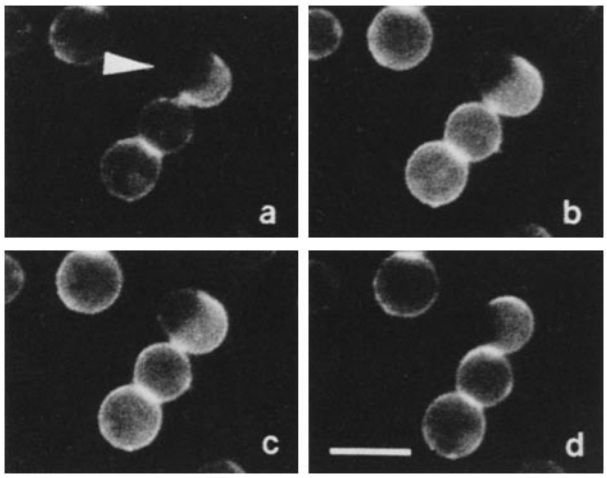

FIGURE 7 Effect of sodium azide treatment on the pattern of R-WGA fluorescence on live sperm. (*a*) Before azide treatment. Spermatids exhibit ring-staining pattern, spermatozoon (top) shows typical asymmetric staining with fluorescence on the cell body much brighter than on pseudopod; (*b*) same field 30 sec after adding 1 mM NaN_3. Note slight increase in fluorescence intensity on spermatozoon pseudopod. (*c*) 90 sec after adding NaN_3. Return of R-WGA to spermatozoon pseudopod complete. Label remains asymmetrically distributed but much less markedly than in (*a*). (*d*) 1 min after washing out azide. Pseudopod has moved and cleared its fluorescence so the cell is as asymmetric as in (*a*). Bar = 5 μm.

The nondirected movement of beads on the surface probably reflects the same membrane movement that produces rotation of tethered spermatids responding to monensin (18). Beads attached to the surface do not move before the cells are treated with monensin. Therefore, their movement is not induced simply by cross-linking of membrane components. It is likely that monensin treatment induces rearrangement of the surface of the cell that is reflected by the movement of attached beads, but the function of this rearrangement is unknown.

The pattern of bead movement reveals four characteristics of this surface rearrangement. First, discrete portions of the membrane are mobile while other portions are stationary. Thus, on a single cell some attached beads moved while others remained stationary. Second, our observation that individual beads were able to move, stop, and start moving again indicates that the rearrangement of the membrane is intermittent rather than continuous. Third, the surface is apparently randomly rearranged because beads wandered over the surface rather than moving in a distinct pattern and did not accumulate at a particular region on the surface. Fourth, as evidenced by the cessation of bead movement, this type of surface rearrangement stops when wild-type cells start extending their pseudopod.

Nearly all *fer-15* mutant spermatids fail to respond to monensin. These cells do not rotate, rearrange their surface, or extend pseudopods. The natural inducer of nematode sperm morphogenesis, mimicked by monensin, is probably a secretion from the male vas deferens which contacts male sperm during mating (10, 15, 29). Sperm from mated *fer-15* mutant males also fail to differentiate (T. Roberts, unpublished observation). Thus, further analysis of these mutant sperm may reveal how nematode spermatids detect the signal to complete morphogenesis.

fer-1 mutant sperm fail to stop bead movement on their cell body after extending pseudopods. We report in a following paper (22) that beads attached to the pseudopods of mature wild-type sperm are transported to the base of the pseudopod but never move onto the cell body. On *fer-1* mutant sperm, beads can move from the cell body to the pseudopod and return. Thus, these mutant sperm are deficient in both stopping membrane rearrangement on the cell body and in preventing membrane movement from pseudopod to cell body. This suggests that these two events may be controlled by the same mechanism. *fer-1* mutant sperm fail to fuse their MO's with the surface (29). Perhaps MO fusion stabilizes the cell body membrane. Without this stabilization the cell may not be able to stop rearranging its surface or establish a barrier to membrane movement between the pseudopod and the cell body.

Just after R-WGA and NBD-PC treated spermatids extend their pseudopods both fluorescent probes were evenly distributed over the surface of the pseudopod. This shows that the extending pseudopod derives at least part of its membrane from the surface of the spermatid. We have not compared quantitatively the intensity of fluoresence on spermatids to that on immature spermatozoa. However, morphometric analyses indicate the surface area of *C. elegans* spermatids and spermatozoa appears to be the same (S. Ward, unpublished observation). Therefore, pseudopod extension does not necessarily require assembly of any new surface membrane. We do not know why beads are excluded from extending pseudopods. It may be that they clear so rapidly that they are gone by the time a pseudopod is recognizable.

The clearance of lectin receptors and lipids from the surface of developing pseudopods, appears to be the same as the movement that continually transports attached beads from the tip of the pseudopod to its base in mature spermatozoa (22). In the present study, the pattern of removal of ferritin-labeled lectin receptors from the pseudopod was similar to the movement of beads on pseudopods. The increased resolution afforded by EM showed that clearance of ferritin-labeled lectin started at the tips of the pseudopodial projections. Finding cells with the distal portion of their pseudopod cleared but with the proximal portion still bearing ferritin-labeled lectin receptors indicates that, like beads, lectin receptors move toward the base of the pseudopod. Beads stopped moving on pseudopods at the junction with the cell body without internalization, movement onto the cell body, or return to the pseudopod. Likewise, we found that ferritin accumulated at the cell-body-pseudopod junction. We never found internalized ferritin nor did we observe an increase in grain density on the cell body after clearance of the pseudopod.

When spermatozoa cleared WGA receptors labeled with fluorescent lectin from their pseudopod we did not observe formation of a bright patch of fluorescence at the cell-body-pseuodopod junction. Possibly such patches, corresponding to the piles of ferritin grains at the base of the pseudopod of

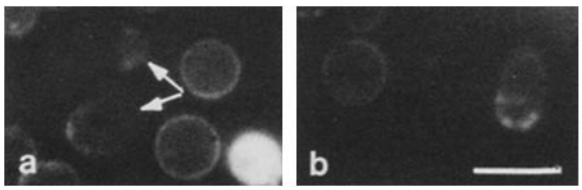

FIGURE 8 Effect of sodium azide treatment on the distribution of NBD-PC in the membrane of live sperm. (*a*) Before azide treatment. Spermatozoa (arrows) show typical asymmetric staining pattern. (*b*) Different field, 11 sec after adding NaN_3. NBD-PC has redistributed on the spermatozoon so that the fluorescence intensity on its pseudopod is about the same as on nearby spermatid. Bar, 5 μm.

mature, lectin-ferritin labeled cells, cannot be resolved by fluorescence microscopy. Alternatively, the cell may be able to internalize lectin-receptor complexes but not larger membrane-bound structures, such as ferritin and latex beads.

That labeled lectin receptors and fluorescent phospholipids are cleared from the pseudopod at about the same rate is of particular importance. Several theories seek to explain directed movements of membrane components such as capping of antigens and lectin receptors on lymphocytes or centripetal transport of receptors or inert particles on amoeboid cells (discussed in reference 14). These theories break down into two groups, one of which attributes movement of selected membrane components to their direct or indirect association with cytoplasmic contractile proteins. The other proposes that directed movement is a manifestation of polar assembly-disassembly of part or all of the cell membrane. These two groups of theories predict different fates for lipids incorporated into the plasma membrane. As pointed out by Bretscher (9), membrane integrated lipids do not span the bilayer and, therefore, cannot associate directly with cytoplasmic proteins so directed movement of membrane lipids must be due to membrane flow, or association with membrane proteins.

Stern and Bretscher (26) demonstrated directed movement of Forsmann glycolipid incorporated into the surface membrane of several types of vertebrate cells after treatment with anti-Forsmann antibody. The authors attributed this movement to selective directed flow of membrane lipids, as originally proposed by Bretscher (9). Likewise, Schroit and Pagano (25) demonstrated antibody-induced capping of a trinitrophenylated derivative of NBD-phosphatidylethanolamine in the surface membrane of lymphocytes. Their report included a convincing demonstration that the lipid was properly integrated into the plasma membrane.

Here we found that NBD-PC was cleared from the surface of the pseudopod at about the same rate as glycoprotein lectin receptors, but not cleared from the cell body of *C. elegans* spermatozoa. The cell body and the fused MO's retained their fluorescence and there was no increase in cytoplasmic fluorescence when the pseudopod membrane was cleared of NBD-PC suggesting that removal of the lipid was in the plane of the membrane.

Our interpretation of the results obtained using NBD-PC depends on the lipid being integrated into the surface membrane and free of association with membrane proteins. Association of vesicular lipids with cell membranes could occur in three ways: sticking of vesicles to the surface of the cell, exchange of lipid between the cell membrane and the vesicle, or actual fusion of the vesicle with the membrane with dilution of vesicle contents in the plane of the membrane (21). Only the latter two of these result in incorporation of lipid into the cell membrane. Several observations suggest that the fluorescence we detected was due to free lipid in the membrane bilayer. The fluorescence of intact NBD-PC vesicles was completely quenched (i.e. solutions of vesicles alone did not fluoresce). Therefore, adherent vesicles should not have contributed to the fluorescence we observed on the cells. Results obtained by fixing or metabolically disrupting asymmetrically labeled spermatozoa indicate that NBD-PC was in the membrane free of restraint. If the lipid were still in vesicles or associated with integral membrane proteins aldehyde fixation should prevent its lateral diffusion. This was not the case because fixation immobilized lectin-receptor complexes but allowed diffusion of NBD-PC into the pseudopod membrane.

Determining the time required to reestablish maximum pseudopod membrane fluorescence after azide treatment of asymmetrically labeled spermatozoa allows us to estimate the diffusion coefficients, D, of either WGA-receptor complexes or NBD-PC in the membrane bilayer. D is approximated from the solution of the diffusion equation for intermixing of two solutes in the surface of a sphere (16): $D = r_0^2/2\,\tau$ where r_0 is the radius of the cell (~ 2 μm) and τ is the half-time of intermixing (~ 40 s for lectin-receptor complex and 8 s for NBD-PC). These values estimate $D \simeq 5 \times 10^{-10}$ cm^2 s^{-1} for lectin-receptor complex and $\geq 2.5 \times 10^{-9}$ cm^2 s^{-1} for NBD-PC. These estimates agree closely with values obtained by fluorescence recovery after photobleaching techniques applied to R-WGA-receptor complex in the surface membrane of *C. elegans* spermatozoa (2×10^{-10} cm^2 s^{-1}; 3) and to NBD-PC in the plasma membrane of V79 fibroblasts ($2.04 \pm 0.47 \times 10^{-9}$ cm^2 s^{-1}; 27). It should be pointed out that WGA binds to several species of membrane glycoproteins and, therefore, our estimate of D represents an average for several different lectin-receptor complexes. Also, the value obtained for NBD-PC is an underestimate because we were unable to accurately determine τ. Nevertheless, the marked difference in D for a protein-protein complex and a lipid and the similarity of our values with those reported elsewhere lead us to conclude that NBD-PC was properly integrated into the sperm plasma membrane and was free to diffuse.

In total, our data strongly favor membrane flow over transmembrane linkage to a cytoplasmic contractile system as the mechanism driving the centripetal movement of membrane components on the sperm pseudopod. Lipids, latex beads, and lectin-receptor complexes are cleared from the pseudopod in the same direction and at approximately the same velocity. Although we cannot rule out transmembrane control of the motion of lectins and beads, we have shown NBD-PC behaves as a properly integrated free lipid in the sperm membrane and, thus, must avoid transmembrane control. Our data do not determine conclusively whether sperm membrane flow occurs by polar assembly-disassembly of lipids alone (9) or by bulk membrane flow (1, 2, 7, 12, 13). We demonstrate in a following paper (22) continuous insertion of lectin receptors at the tip of the sperm pseudopod followed by their directed movement toward the base of the pseudopod. According to the lipid flow theory, membrane proteins move by being trapped in the lipid stream. Reinsertion of this class of proteins is not required (but not precluded). By definition, the bulk membrane flow theory requires that clearance of a class of receptors be accompanied by their immediate reinsertion in the cleared zone. Therefore, our data support the bulk membrane flow theory of membrane movement.

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