**Ultrastructural study of the Sex Ratio Organism (SRO)
transmission into oocytes during oogenesis in
Drosophila melanogaster.**

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ABSTRACT

The transmission process of Sex Ratio Organisms (SROs) into oocyte during oogenesis in *Drosophila melanogaster* was studied by electron microscopy. SROs break through a tunica propria, a non-cellular membrane surrounding the egg chamber, and move toward oocytes passing through the intercellular space of the follicle cell layer at previtellogenic stages. After reaching at the surface of an oocyte, they are incorporated into the ooplasm by pinocytosis with progress of vitellogenesis. By the final stages of oogenesis, most SROs become infolded in intracellular vesicles and yolk granules followed by transfer to the interior of the oocyte.

1. INTRODUCTION

The Sex-Ratio Organism (SRO) infecting *Drosophila* is a transovarially transmitted spiroplasma (Class *Mollicutes*, Order *Mycoplasmatales*) that causes son-specific lethality (review, Williamson and Poulson 1979). They are helical, motile, cell wall-free prokaryotes of 0.15 μm in diameter and 5–6 μm in length (Williamson 1969) and found abundantly in the adult hemolymph (Poulson and Sakaguchi 1961; Sakaguchi and Poulson 1961). The injection of the SRO-laden hemolymph into normal females of the same species or other *Drosophila* species generally results in persistent infection and show male-killing effects (Williamson and Poulson 1979, and references therein). SROs kill single-X individuals regardless of their phenotypic sex (Sakaguchi and Poulson 1963; Miyamoto and Oishi 1975; Watanabe 1975; Fujihara, Kawabe and Oishi 1978). Recently Yamada, Watanabe and Koana (1985) reported there is no single region on the X chromosome that makes, when duplicated, males resistant to the SRO. Attempts to cultivate the SROs *in vitro* and to characterize genomes of SROs and SRO viruses in molecular terms have just begun (Hackett *et al.* 1986; Ueda, Koana and Miyake 1987; Cohen, Williamson and Oishi 1987).

The actual process by which SROs are transmitted into oocytes during oogenesis has not yet been elucidated, though transovarial transmission is known to occur from earlier studies (e.g. Malogolowkin and Poulson 1957; Malogolowkin 1958). For clarification of this point, we analyzed the mode of transmission of

SROs into oocytes during oogenesis in ORNSR flies (an Oregon-R stock carrying the SRO derived from *D. nebulosa*, NSRO) by electron microscopy.

The data of the present study demonstrate SROs to migrate into an oocyte by pinocytosis at the start of vitellogenesis and then to become enclosed in intracellular vesicles and in yolk granules of the oocyte.

2. MATERIALS AND METHODS

The fly stock used was an ORNSR (an Oregon-R stock carrying NSRO), a strain established previously and was kindly supplied by Dr. Sakaguchi of Kyushu University. The stock was maintained as described by Oishi (1971). An Oregon-R stock without infection was used as control. Ovaries and embryos were prepared from adult females 10–12 days-old. Dissected egg chambers and dechorionated embryos were prepared for transmission electron microscopy (TEM) according to the method of Niki (1984). Some egg chambers were post-fixed with 3% KMnO_4 in stead of 2% osmic acid so as to obtain a detailed visualization of the membrane structure. Egg chambers were classified according to King (1970).

3. RESULTS

(1) SROs at previtellogenic stages.

Egg chambers of ORNSR females develop quite normally, showing no morphological abnormalities or developmental delays at any stage (Fig. 1).

No SROs are found in oocytes or nurse cells at any previtellogenic stages (Fig. 2). However, they are abundantly present between the enveloping peritoneal sheath and follicle cells (Fig. 3). It should be noted that absence of SROs outside the peritoneal sheath in the figure is a result of their being washed away during preparation for transmission electron microscopy (TEM), though numerous SROs are present in the hemolymph of living female abdomens.

Fig. 4 shows SROs breaking through a tunica propria, a non-cellular membrane surrounding an egg chamber of stage 5 (previtellogenic stage). They then populate the intercellular space between the tunica propria and follicle cell layer (Fig. 5). TEM pictures obtained from KMnO_4 post-fixed materials confirmed SROs not to penetrate the nurse cells or follicle cells but remain in the intercellular space of an egg chamber (Fig. 5a). The SROs move toward an oocyte through the space (Fig. 5b) to become abundantly present along the oocyte surface up to the beginning of vitellogenesis (Fig. 6).

(2) SROs during vitellogenesis.

A brief description of normal events of vitellogenesis is given below to facilitate understanding of the subsequent transmission of SROs (see King (1970) and Mahowald and Kambyzellis (1980) for detailed description). At stage 8, an oocyte

Fig. 1: Photomicrographs of $1\text{ }\mu\text{m}$ -thick sections of egg chambers of 12 days-old ORNSR flies at stages (King 1970) of 3-4 (a), 8 (b), 10 (c) and 12 (d), respectively. (o: oocyte; nc: nurse cells; fc: follicle cells). (b) At stage 8 vitellogenesis commences. (c) At stage 10 the size of an oocyte becomes approximately half of an egg chamber and the regional differentiation of the follicle cells takes place; the posterior follicle cells become columnar. (d) At stage 13 an oocyte reaches the maximum volume and is separated from the follicle cell layer by the vitelline membrane. Nurse cells are seen to degenerate. Bar indicates $10\text{ }\mu\text{m}$.

Fig. 2: A transmission electron micrograph (TEM) of the germ-line cells before differentiating into nurse cells and an oocyte from an ovary of an ORNSR female at stage 3-4. Note that the SROs are absent in these cells. Bar indicates $1\text{ }\mu\text{m}$.

Fig. 3: A TEM of the surface of an egg chamber at stage 5. Note that the SROs, characterized by the presence of electron-dense fibrils in the bodies, are abundant between an enveloping peritoneal sheath (ps) and follicle cells (fc). The follicle cell layer is surrounded by a non-cellular membrane, tunica propria (tp). Bar indicates 1 μ m.

Fig. 4: Higher magnification of the tunica propria of an egg chamber at stage 5. Several SROs are seen to be penetrating the tunica propria. Insert: an SRO has just penetrated the tunica propria. Bar indicates 1 μ m.

commences vitellogenesis. Most materials synthesized in the nurse cells are transported through interconnecting cytoplasmic bridges, ring canals, into the developing oocyte, whereas other materials produced in different tissues (e.g. vitellogenin) are incorporated into the oocyte by pinocytosis. Pinosomes (sites of

Fig. 5: SROs in the follicle cell layer at stage 5-6. (a) An egg chamber is post-fixed with KMnO_4 (see Materials and Methods) and thus the SROs stain darkly. Note that the SROs do not penetrate cell membrane of the follicle cells but remain in the intercellular space. (b) The SROs are found to be migrating toward an oocyte through the intercellular space of the follicle cell layer. Bar indicates 1 μm .

pinocytosis activity) become prominent at the plasma membrane over the entire surface of the growing oocyte as vitellogenesis proceeds. Intracellular vesicles in which vitellogenin and other substance have been infolded start fusing with each other and move toward the interior of the oocyte. At stages 9-10, regional differentiation of follicle cells takes place. Most of the follicle cells move posteriorly and become columnar. Then they take on the form of vitelline bodies (precursor of the vitelline membrane) in the area facing the oocyte. Soon after this, the vitelline bodies coalesce and the oocyte becomes to be separated from the follicle cell layer by the vitelline membrane. At stages 12-13, the oocyte attains maximum size, nurse cells degenerate and follicle cells synthesize the chorion outside the vitelline membrane.

In the egg chamber of an ORNSR female, numerous SROs migrate up to the periphery of the oocyte until the beginning of vitellogenesis (Fig. 6). Some SROs are seen trapped in the pinosomes and others in intracellular vesicles at the periphery of the oocyte (Figs. 6 and 7). Still others are seen in the ooplasm free of vesicular membrane (Figs. 6c and 8a). Since no SROs are found in the ooplasm before vitellogenesis, it is most probable that they migrate in the ooplasm only by pinocytotic activity at these stages. At later stages of vitellogenesis (stage 10 and thereafter), SROs frequently appear attached to the surface of yolk granules

Fig. 6: Posterior region of an egg chamber at stage 8. (a) The SROs are no longer seen in the intercellular space of the columnar follicle cell (fc) layer. (b) Numerous SROs are seen at the surface of an oocyte (o). (c) Some SROs (arrowheads) are found to enter in an oocyte and are infolded in intracellular vesicles in the ooplasm. y: yolk sphere. Bar indicates 1 μ m.

and/or infolded in the yolk granules (Fig. 8). Noticeably, there are cases of their occupying most of the interior of the yolk granules (Fig. 8b). It should be noted that more SROs are present in larger yolk granules than in smaller granules. It thus appears that SROs enter yolk granules while intracellular vesicles fuse with each other to form larger granules. The yolk granules and intracellular vesicles enclosing the SROs are then carried within the oocyte (Fig. 9).

Few SROs are found in intercellular spaces between the columnar follicle cells (Fig. 6). SROs simply may not be able to penetrate into these spaces because of the close adherence of the columnar follicle cells to each other. Once the vitelline

Fig. 7: An egg chamber at stage 10. The vitelline bodies (vb) (precursor of the vitelline membrane) are formed at the intercellular space between the follicle cells and an oocyte. The SROs are seen to be trapped by the pinosomes (p) and enclosed in intracellular vesicles in the ooplasm (arrowheads). SROs are not found in the area between the vitelline bodies and the follicle cells. Bar indicates 1 μ m.

Fig. 8: SROs in the oocyte of stage 10. (a) Some SROs are apparently freely swimming about in the cytoplasm (arrowheads) but many are found to be attaching the surface of yolk granules (arrowheads) and (b) are infolded in the interior of the yolk granule. Bar indicates 1 μ m.

Fig. 9: A mature oocyte. SROs (arrowheads) are seen most frequently in the intracellular vesicles and the yolk granules at the inner region of an egg but few at the peripheral ooplasm. vm, vitelline membrane. Bar indicates 1 μ m.

Fig. 10: An egg after fertilization. SROs are found to be present in the cytoplasm free of vesicular membrane. Bar indicates 1 μ m.

bodies coalesce to seal the entire surface of the oocyte, the oocyte is separated from the follicle cell layer (Fig. 7). Consequently, SROs outside the vitelline membrane are no longer able to migrate to the periphery of the oocyte. At the final stages of oogenesis (stages 13 and 14), only a few SROs are found in the oocyte periphery (Fig. 9). It is thus evident that most SROs at the surface of oocytes from early vitellogenic stages have finished migrating into the ooplasm.

After fertilization, SROs leave the yolk granules and move about in the ooplasm free of vesicular membranes (Fig. 10).

4. DISCUSSION

Present study shows SROs to become incorporated into vitellogenic oocytes by pinocytosis following penetration of the tunica propria. Since SROs are absent in nurse and follicle cells at all stages of oogenesis, even though motile, it may be concluded that they are not capable of penetrating cellular membranes. SROs can migrate to the surface of a developing oocyte passing through the interfollicle cell space. However, when follicle cells become columnar, they can no longer pass through the interfollicle cell space, nor can they penetrate the vitelline

membrane. The number of SROs that can be incorporated into an oocyte is thus restricted. The SR syndrome showing lethality depends on the age of the female parents (Oishi 1971). This may be related in part difference in time required for an immature oocyte to mature, and in part to the difference in the pionocytotic activity of oocytes, in females of different ages. Detailed studies may be expected to produce some insights into the physiology of developing oocytes of females of various ages. It may also be possible to estimate the actual number of SROs incorporatable by an egg of a female parent and the threshold number of SROs required for male lethality during development.

Transovarially transmitted bacteria are known in several insects: for example, in mosquito *Culex tarsalis* (Kellen and Wills 1962) and *C. salinarius* (Andreadis and Hall 1972). It should be of considerable interest to see if the transovarial transmission in these cases and others also takes the same or similar courses as in the SRO. Ehrman and Kernaghan (1971) reported that mycoplasma-like organism causing hybrid sterility in *D. paulistorum*, upon infection by injection into abdominal hemocoel, were observed to appear in nurse cells, oocytes as well as follicle cells of the developing ovaries. Apparently, the transmission of the mycoplasma-like organisms is quite different from that of the SRO, since the latter was never observed in cells other than oocytes.

SROs begin leaving yolk granules and intracellular vesicles after fertilization. Preliminary observations show that some are trapped in blastoderm cells but most are distributed between the blastoderm cell layer and central yolk region at the blastoderm stage. Developmental feature of the SR syndrome to contributing to male lethality was first analyzed by Counce and Poulson (1962) who found embryonic lethality to start at very early stages before gastrulation. Primordial mesodermal and/or nervous tissues have been suggested as a possible candidate for the primary target of SROs on the basis of studies conducted on gynandromorphs (Tsuchiyama, Sakaguchi and Oishi 1978). Among primary cell cultures from single embryos nerve cells, but not mesoderm-derived muscle cells, are severely affected by the SRO (Koana and Miyake 1983). Further analysis should be carried out to elucidate the causality of SRO behavior and male lethality during development.

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