Squash preparations of their heads were prepared for FA examination, as described previously.[2] Their abdomens were then removed, and either the ovaries were dissected out or frozen sections were prepared. For fresh tissue preparations, the ovaries were removed intact and were transferred to a drop of phosphate buffered saline, pH 7.4 (PBS), on a glass microscope slide. The ovarioles and other associated structures were then carefully separated under a dissecting microscope to allow better visualization. To prepare frozen sections, the entire abdomen was embedded in Tissue-Tek O.C.T. compound (Ames Company, Miles Laboratories, Inc., Elkhart, Indiana) and then quick-frozen in a mixture of Dry Ice and alcohol. Serial sections of the frozen abdomen were later cut on a cryostat at −15°C and were mounted on clean microscope slides. The prepared slides were then dried at room temperature, fixed in cold acetone for 10 min, and stained with an anti-San Angelo FA conjugate for 60 min at 37°C. The method used to prepare the antibody conjugate was that reported earlier by Kuberski and Rosen.[2] Evan's Blue dye was added to the conjugate at a dilution of 1:10,000 to counterstain the ovarian tissue and to increase the contrast with viral antigen.

Examination of specimens

Mosquito head squash and ovary preparations were stained by a direct FA technique as outlined previously.[2] After staining, the slides were washed in PBS for 10 min and then were mounted with a 1:1 mixture of PBS and glycerol. All specimens were examined with a Leitz Dialux fluorescent microscope equipped with an HBO 200W high pressure mercury lamp, a BG-38 heat absorbing filter, a BG-12 exciting filter, and a K510 suppression filter. Photographs were taken with a Leitz Orthomat automatic camera, using Ektachrome-400 color film.

REVIEW OF DEVELOPMENT OF THE MOSQUITO OVARIOLE

In a newly emerged female mosquito, the ovaries are located bilaterally in the area of the fifth abdominal segment. Each ovary consists of 50–100 ovarioles[3] which are clustered together around the internal oviduct, appearing like a bunch of tiny grapes. The ovariole is the basic unit of the female reproductive system. Descriptions of its anatomy have been given by others.[4–7] Figure 1 shows the relationship of structures in a mosquito ovariole and illustrates follicles in various stages of development. The follicular epithelium, nurse cells and oocyte together make up the follicle.

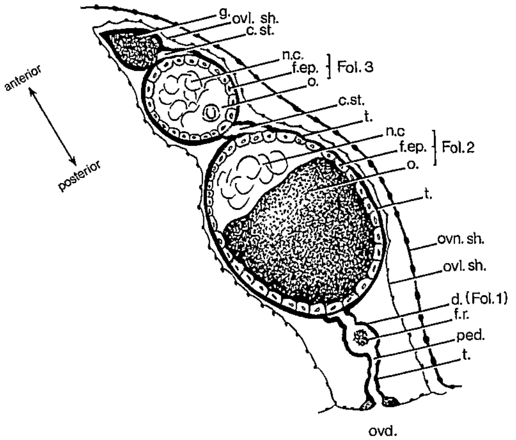
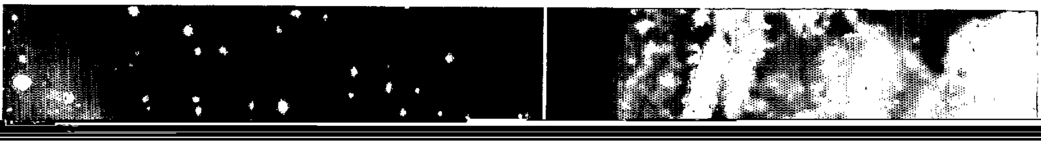
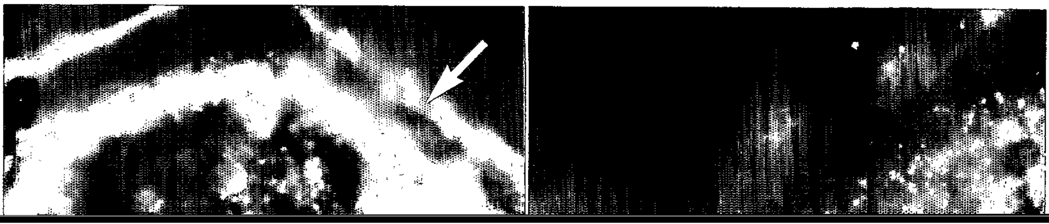


FIGURE 1. Schematic drawing showing the relationship of structures in a mosquito ovariole (after Bertram[4]). c. st., connecting stalk; d., dilatation representing former primary (first) follicle; f. ep., follicular epithelium; Fol.[2], Fol.[3], second and third follicles; f.r., follicular relic; g., germarium; n.c., nurse cells; o., oocyte (stippling indicates yolk granules); ovd., oviduct; ovl. sh., ovariole sheath; ovn. sh., ovarian sheath; ped., pedicel; t., tunica.

The progressive changes which occur in the ovarioles during oogenesis have been divided by Christophers[6] into five arbitrary phases. Stage 1 is seen only in the newly emerged female mosquito. In this phase, the follicle is spherical in shape and about 60–80 μ in diameter.[4] Yolk granules are absent and the nurse cells and oocyte are clearly visible.[6,7] Stage 2 is seen in the mosquito several days after emergence but before it has taken a blood meal. The follicle in this phase is oval in shape and about 100 μ in diameter.[4] Yolk granules begin to appear around the nucleus of the oocyte.[6,7] At this stage, the oocyte is much larger than the nurse cells and occupies the entire posterior half of the follicle. Further development of the follicle stops at this stage until the mosquito takes a blood meal.

Within 24 hours after the blood meal, there is a marked increase in the number of cells in the follicular epithelium.[5] Stage 3 begins when the oocyte nucleus becomes obscured by the accumulating yolk.[4,6,7] At this phase, the oocyte takes up most of the follicle which is still oval and about





stage 1 of ovarian development, viral antigen was seen only in the oviduct, interstitial cells and ovariole sheath. Antigen did not appear in the follicular epithelium, oocyte or nurse cells until stage 2. However, after the blood meal there was a rapid accumulation of viral antigen in the developing oocytes. The quantity of antigen which accumulated in the follicles between stages 2 and 5 was impressive (Fig. 4–7) and occurred in a period of about 72 hours, indicating rapid virus replication. It is surprising that so much virus could be present without causing some harm to the egg. It is possible that some of the fluorescent material represents noninfectious or incomplete virus; but previous studies have indicated that SA virus infection does not affect the survival or fecundity of *Ae. albopictus.*[8] In general, most arboviruses do not appear to be harmful to their arthropod hosts. These observations support previous suggestions[1] that SA virus probably has a symbiotic relationship with the mosquito.

Another interesting finding was that the number of infected follicles varied within different female mosquitoes. For example, in some of the insects 100% of the follicles were infected; in others only 50 or 60% of stage 5 follicles contained viral antigen. In the latter group, negative follicles were scattered randomly throughout the ovary despite the presence of large amounts of viral antigen in the adjacent follicles and oviduct (Fig. 5). This is compatible with previous observations that the filial infection rate of SA virus among *Ae. albopictus* females in the chronically infected line is not always 100% and shows considerable individual variation.[1] The explanation for why some follicles become infected and others do not, within the same female mosquito, is unclear.

It should be emphasized that the presence of virus or viral antigen in a mosquito's ovary does not necessarily mean that the agent will enter the oocytes and infect the progeny. For example, FA and electron microscopic studies of mosquitoes experimentally infected with dengue,[9] Japanese encephalitis,[10] St. Louis encephalitis,[11] and Zika served in the follicles. Furthermore, transovarial transmission of these viruses in mosquitoes occurs at very low rates and in only some species.[8, 12, 13] The mechanism which determines whether or not an arbovirus will be transovarially transmitted in mosquitoes is unknown. It may be genetic in part, but it is difficult to explain why some follicles are infected and others are not in the same mosquito, since all follicles in a given female are genetically identical.

In an earlier study of the SA virus chronically-infected *Ae. albopictus* line, Tesh and Shroyer presented data indicating that the virus was transmitted from generation to generation by maternal (cytoplasmic) inheritance.[1] It was suggested that SA virus infects the germ cells (oogonia) of some female mosquitoes, producing a stabilized infection analogous to the behavior of sigma virus in *Drosophila melanogaster.*[1] In the present study, viral antigen was not observed in the germarium at any stage of ovarian development. However, antigen appeared in the follicular epithelium, nurse cells, and oocytes (structures which originate from the germarium) during the second and third stages of ovarian development. It is possible that SA virus may be present in the germarium and germarial-derived tissues in some undetectable form and that certain physiologic changes associated with feeding (i.e., vitellogenesis or proliferation of the follicular epithelium) stimulate virus replication in the latter tissues. Alternatively, it is also possible that the virus enters the oocyte through the follicular epithelium from surrounding nonovarian structures, a method used by a number of other transovarially transmitted endosymbionts of insects.[14–17] For example, the fat body also contained a large amount of viral antigen. Since vitellogenic protein is synthesized in the insect fat body, transported through the hemolymph, and deposited in the oocyte,[18] it is possible that the virus and/or viral antigen might enter by the same route. Regardless of how the virus gets into the oocyte, the hypothesis that SA virus is transmitted by cytoplasmic inheritance

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