



THE LOCATION OF SAN ANGELO VIRUS IN DEVELOPING OVARIES OF TRANSOVIARIALLY INFECTED *Aedes* *albopictus* MOSQUITOES AS REVEALED BY FLUORESCENT ANTIBODY TECHNIQUE*

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Abstract. *Aedes albopictus* adult female mosquitoes, transovarially infected with San Angelo (SA) virus, were examined by fluorescent antibody technique during various stages of ovarian development to determine how the virus enters the egg. Upon emergence from the pupal stage, viral antigen was observed only in the oviduct and ovariole sheath. By the 4th day of adulthood, it was visible in the follicular epithelium, oocytes and nurse cells of the primary follicles. In the 72-hour period between the ingestion of blood and oviposition, there was a marked increase in the amount of viral antigen in the oocyte, indicating rapid virus accumulation. After oviposition, SA viral antigen was also seen in the secondary ovarian follicles. The observed sequence of infection of the mosquito ovariole with SA virus is analogous to that described with certain endosymbionts of insects.

Recently, Tesh and Shroyer described a line of *Aedes albopictus* mosquitoes, chronically infected with San Angelo (SA) virus, which transovarially transmitted the virus from generation to generation to a high percentage of their progeny.¹ It was postulated that most of the female mosquitoes in this line had oogonial (germ cell) infection, analogous to that described with sigma virus in *Drosophila melanogaster*.¹ It was also suggested that this might be a mechanism by which some arboviruses are maintained in nature.¹

In the present study, ovaries from *Ae. albopictus* of the aforementioned chronically infected line were examined by fluorescent antibody (FA) technique during various stages of development to determine the location of SA virus. This paper reports our observations and attempts to describe how the virus enters the developing oocytes.

MATERIALS AND METHODS

Mosquitoes

The females used in this study were from the previously described,¹ chronically infected *Ae.*

albopictus line. They were maintained by random brother-sister matings and represented the 16th and 17th consecutive transovarially infected generations. Approximately 75% of the females in these generations were infected. Mosquito pupae were placed in individual cotton-plugged tubes, containing a small amount of water, so that the sex and time of emergence of the adults could be determined. This procedure also prevented copulation with their infected siblings and possible venereal transmission of virus. After emergence, the adult females were placed in small marked cardboard containers and were maintained on 10% sucrose solution at 28°C. About 18 infected mosquitoes were killed and examined every 24 hours from the day of emergence until the 9th day of adulthood. Noninfected female *Ae. albopictus* of the same age and stage of ovarian development were also examined each day as controls. On the 4th day after emergence the surviving mosquitoes (infected and control) were fed on a mouse to initiate egg development.

Preparation of specimens

On the day of examination, the mosquitoes were chilled in an ice bath and then decapitated.

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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 ovarioles³ 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

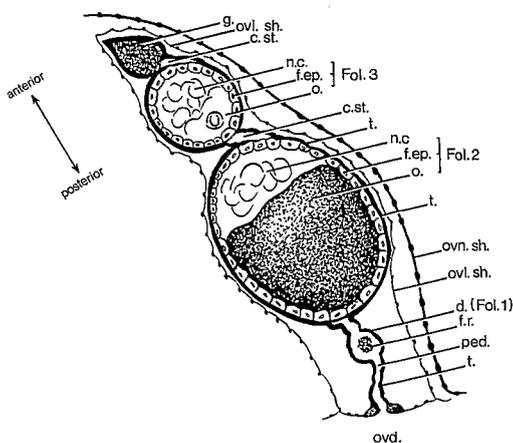
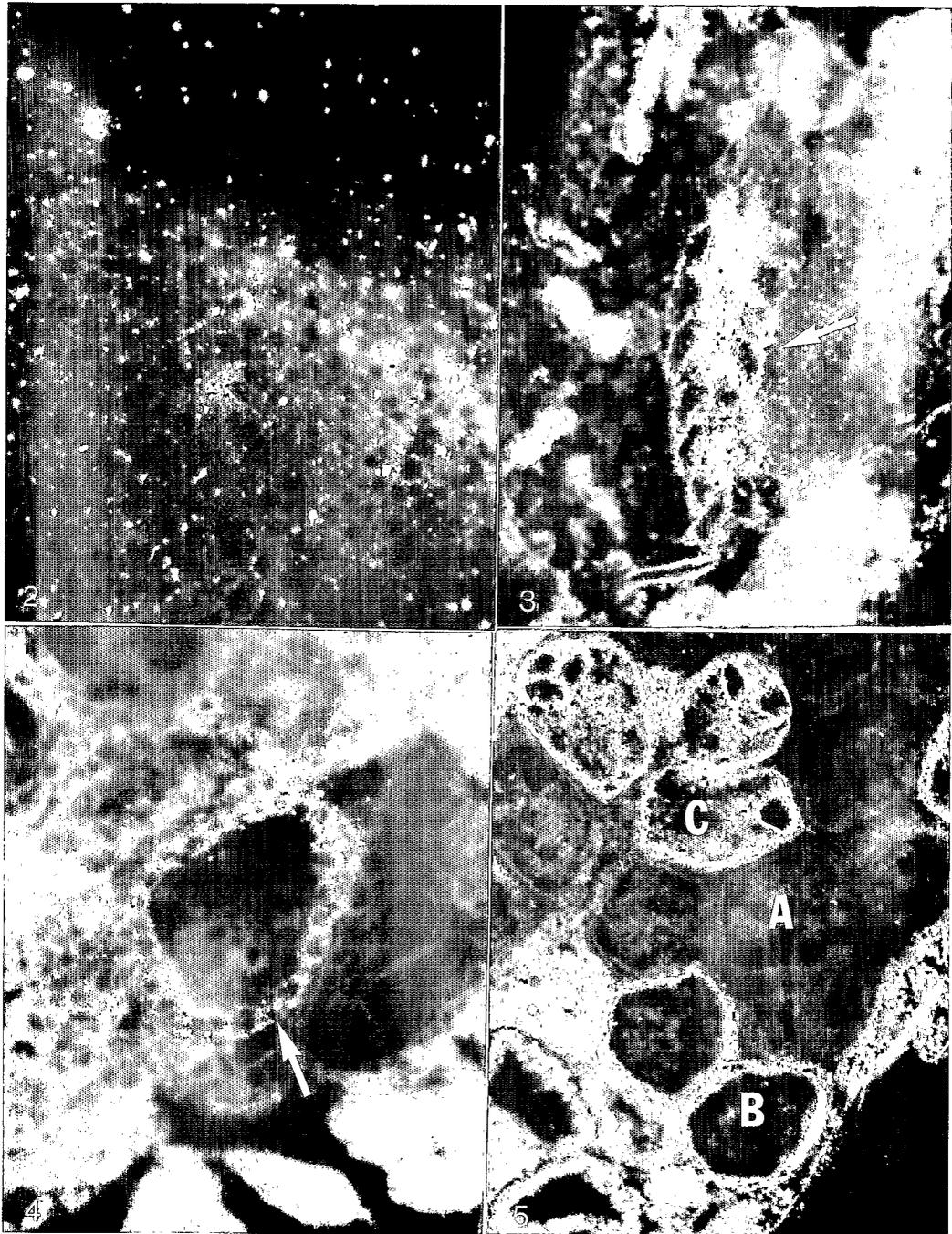


FIGURE 1. Schematic drawing showing the relationship of structures in a mosquito ovariole (after Bertram³). c. st., connecting stalk; d., dilatation representing former primary (first) follicle; f. ep., follicular epithelium; Fol.², Fol.³, second and third follicles; f. r., follicular relic; g., germarium; n. c., nurse cells; o., oocyte (stippling indicates yolk granules); ovd., oviduct; ovl. sh., ovariole sheath; ovn. sh., ovarian sheath; ped., pedicel; t., tunica.

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.

The progressive changes which occur in the ovarioles during oogenesis have been divided by Christophers⁶ 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



FIGURES 2-5. 2. Head squash preparation of a transovarially infected *Ae. albopictus*, showing the appearance of San Angelo viral antigen by the fluorescent antibody technique. Viral antigen appears as multiple small fluorescent foci (white spots in photograph) scattered throughout the tissues. $\times 625$ approx. 3. A longitudinal section through the abdomen of a newly emerged, transovarially infected female mosquito. Viral antigen is present in most of the abdominal organs, but the ovaries (arrow) are the brightest staining structures in the abdomen. $\times 156$ approx. 4. Frozen section through an infected stage 2 follicle (arrow). Viral antigen is present in the follicular epithelium but is not visible in the oocyte or nurse cells. $\times 625$ approx. 5. Longitudinal section through stage 3 follicles in the ovary

200 μ long.⁴ In stage 4, the follicle becomes elongate and assumes the size (0.5–1.0 mm) and shape of the future egg.^{4, 6, 7} The yolk-laden oocyte now occupies almost all of the follicle and the degenerating nurse cells are extruded through the micropyle. Stage 5 occurs when the chorion covers the entire egg.^{4, 6, 7} This stage ends with oviposition.

After the blood meal, as the first follicle enters stage 3, a second follicle separates from the germarium and begins to develop.⁴ The new follicle grows to stage 2 by the time the first egg is laid. It remains quiescent in this stage until the next blood meal, when the entire process is repeated again.

RESULTS

Figure 2 shows a head squash preparation of a transovarially infected *Ae. albopictus* female, illustrating the appearance of SA viral antigen by FA technique. The antigen appears as multiple small fluorescent foci (white spots in photograph) scattered throughout the tissue.

In transovarially infected female mosquitoes examined within 12 hours of emergence, SA viral antigen is seen in most of the abdominal organs. Upon emergence, the ovaries are quite small; nevertheless, in longitudinal sections of infected mosquitoes the ovaries are the brightest staining structures in the abdomen and are easily recognized (Fig. 3). At this stage of ovarian development (stage 1), viral antigen is concentrated in the ovarian sheath, oviduct and interstitial cells between the ovarioles. Viral antigen is also present in the ovariole sheath, but it is not seen within the germarium, follicular epithelium, oocyte or nurse cells.

During stage 2 of ovarian development, the amount of antigen in the ovariole sheath increases. Because of the intensity of fluorescent material surrounding infected follicles at this stage, it is not possible to visualize the structures within the follicles in whole preparations. However, in frozen sections viral antigen can be seen in the follicular epithelium as well as in some

nurse cells and oocytes. The appearance of infected follicles in this and in stage 3 varies. In some follicles, antigen is seen only in the follicular epithelium (Fig. 4); in others, it is visible in the follicular epithelium, nurse cells and oocytes (Fig. 5). For this reason we suspect that the former follicles represent an earlier stage of infection and that eventually their oocytes and nurse cells would also be infected.

In stage 3 of ovarian development (shortly after a blood meal), the follicles begin to grow rapidly in size and yolk material quickly accumulates within the oocytes. The rapidly enlarging oocyte now occupies most of the space within the follicular epithelium, squeezing the nurse cells into one corner of the follicle (Fig. 5). At this stage, the follicular epithelium stains very intensely and appears as a bright fluorescent ring encircling the follicle. (Fig. 6).

The fourth stage of ovarian development is quite transient and was not observed.

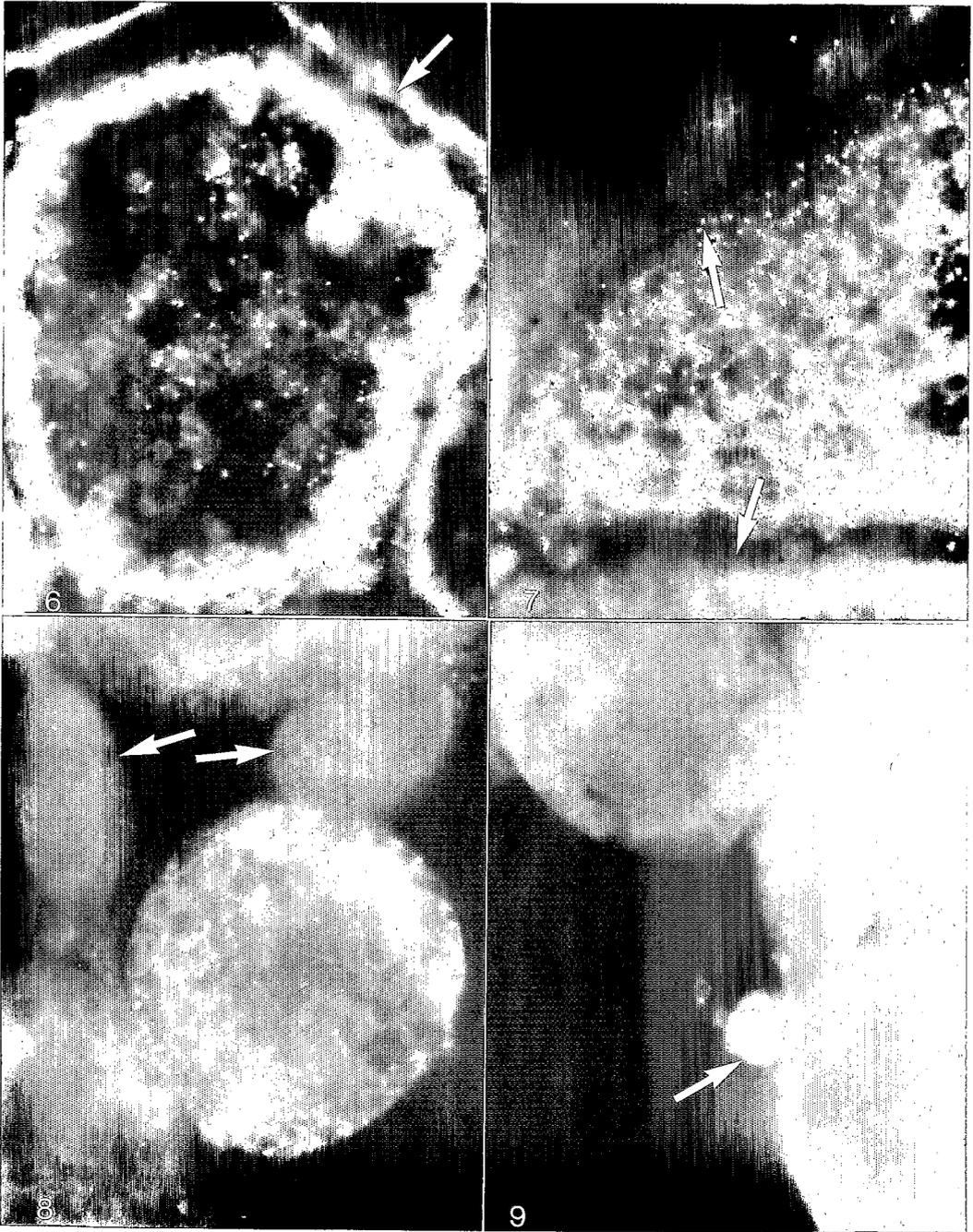
In stage 5, the ovary occupies most of the abdomen. The opaque chorion (egg shell), which is secreted by the follicular epithelium, now completely covers the oocyte. In whole preparations of infected stage 5 follicles, viral antigen can only be seen in the thin ovariole sheath which surrounds the chorion, since the conjugate does not penetrate the thick wall. In frozen sections, however, a large amount of viral antigen is seen within the mature oocytes (Fig. 7). At this point, the eggs are ready to be laid.

After oviposition, the secondary follicles are readily seen in the ovary (Fig. 8). These are similar in appearance to the infected, stage 2 primary follicles described previously. At this time, the dilatation and follicular relic in the pedicel (at the site where the primary follicle had developed) are also visible (Fig. 9).

DISCUSSION

Results of this study suggest that SA virus gains entry into the developing follicle by direct extension from the surrounding ovarian tissues. During

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of an infected mosquito. This photograph illustrates the three types of follicles seen simultaneously in some specimens. Several follicles are uninfected (A), two have viral antigen only in the follicular epithelium (B), and three have antigen in the follicular epithelium, oocyte and nurse cells (C). The latter three follicles show how the nurse cells are squeezed into the anterior corner of the follicle by the developing oocyte. $\times 156$ approx.



FIGURES 6-9. 6. Frozen section through an infected stage 3 follicle. The follicular epithelium (arrow) at this stage stains very intensely. Antigen is also seen in the oocyte, which now occupies most of the follicle. The granular material within the oocyte is yolk. $\times 625$ approx. 7. This photograph shows a section through an infected stage 5 follicle. The chorion (arrows) now surrounds the entire follicle and appears to be antigen-free; but multiple fluorescent foci (viral antigen) are seen within the oocyte. $\times 625$ approx. 8. Whole mount of an infected second follicle in a parous mosquito 8 days after emergence and 1 day after oviposition. At this time, the second follicle has developed to stage 2 and appears like the primary follicle did at the same stage. Viral antigen is present in the ovariole sheath and possibly in the follicular epithelium. Two germaria (arrows) are visible above the infected

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 suggestions¹ 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 (M. Cornet, unpublished data) viruses have demonstrated viral particles or antigen in the oviduct and ovariole sheath, yet these agents were not ob-

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 germarium-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 within the maternal line is still valid. However, further studies are necessary to elucidate the exact mechanism involved.

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follicle. $\times 625$ approx. 9. This photograph shows the dilatation in the pedicel of a parous infected mosquito 10 days after emergence and 2 days following oviposition. Viral antigen is seen in the follicular relic within the dilatation (arrow). The pedicel is connected anteriorly to the developing secondary follicle. $\times 625$ approx.

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