Pathway of Infection of Mosquito Iridescent Virus

I. Preliminary Observations on the Fate of Ingested Virus

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Mosquito iridescent virus (MIV) is ingested in large amounts by first- and second-instar *Aedes taeniorhynchus* larvae without causing a high rate of infection. Electron microscope studies have been undertaken to determine the fate of ingested virus. Preliminary observations suggest that most, if not all, ingested particles are degraded shortly after entering the midgut. MIV and other virus particles employed in this study were apparently unable to penetrate the peritrophic membrane; consequently, none was observed inside, or in contact with, midgut epithelial cells.

Subsequent to the discovery of a mosquito iridescent virus (MIV) in *Aedes taeniorhynchus* by Clark et al. (3), considerable speculation arose concerning the feasibility of its use in biological control of mosquitoes. Unfortunately, however, it is now apparent that only low rates of transmission of this virus can be normally obtained (3, 8, 18). Whereas certain kinds of insect viruses, especially the occluded viruses, are highly infectious when administered per os (2, 5, 10, 13), MIV is not. As part of a general study of insect virus invasion pathways, which previously emphasized occluded viruses (16), we recently also became interested in the uptake by mouth of the icosahedral cytoplasmic deoxyriboviruses, which constitute a large group of viruses found not only in insects but also in many other organisms; MIV appears to belong to this group (15a). Specifically, we wished to find possible explanations for the relative difficulty experienced in attempts to transmit this type of virus per os (14).

A. taeniorhynchus larvae appear to be most susceptible to MIV during the first or second instars, or both, although gross symptoms of disease are not apparent until the fourth (8, 18). As first-instar larvae are extremely small (<1 mm in length at time of hatching), it occurred to us that if such larvae were to ingest a sufficient quantity of virus particles, the fate of these particles could be readily determined by means of electron microscopy. A single longitudinal section would be expected to include much of the alimentary canal and representative portions of most other tissues. This approach has yielded some interesting and unexpected observations which are discussed in relation to mechanisms of virus infection and factors affecting host susceptibility.

MATERIALS AND METHODS

Insects. Eggs of *A. taeniorhynchus* were obtained from T. B. Clark and D. B. Woodward, U. S. Dept. of Agriculture, Lake Charles, La. Mosquito larvae were reared by the method described by Woodard and Chapman (18).

Viruses. A sample of "regular" MIV (3, 12) was also obtained from the above source and was used to infect more larvae (18). Infected larvae were triturated in 0.05 M phosphate buffer (pH 7.2), containing 1.0% L-ascorbic acid, with a 1-ml tuberculin syringe to release virus particles. MIV was purified by density gradient centrifugation (15,000 rev/min, 20 min, 5°C) through linear 10 to 50% (w/v) phosphate-buffered sucrose gradients. Only the bottom (main) band (11) was collected for experiments described here. Virus inocula were maintained at 5°C until use.

The purification of *Trichoplusia ni* granulosis virus capsules has been described in detail (17).

A lyophilized preparation of purified nuclear polyhedrosis virus of *Spodoptera frugiperda* was a gift from John Hamm.

Tipula iridescent virus (TIV) purified from its natural host, *Tipula paludosa*, was donated by K. M. Smith.

Of all the viruses used in this study, only MIV was found to be infectious for *A. taeniorhynchus* larvae.

Feeding experiments. *A. taeniorhynchus* eggs were hatched in batches of approximately 100 in small volumes of deoxygenated (boiled) water. During or shortly after eclosion, an equal volume of virus suspension containing approximately 1 mg of virus per ml was added. First-instar larvae were allowed to feed in the virus suspensions overnight at 28 to 29°C, whereupon larvae were either fixed for electron microscopy or reared to determine the number infected. Feeding experiments were also performed with viruses fixed as described below, except that exhaustive dialysis of the virus suspensions versus phosphate buffer or distilled water was performed. In some cases, fixed *T. ni* granulosis virus capsules were mixed with unfixed iridescent viruses to provide a marker. Most experiments

included a few second-instar larvae for purposes of comparison.

Electron microscopy. Larvae in small plastic dishes were placed on blocks of dry ice in a closed container and anesthetized by a combination of CO₂ and cold temperature. They were then processed for examination in the electron microscope. Initially, it was found that such larvae, if intact, could not be adequately fixed. This problem was overcome either by making a slit in the head capsule, by decapitating, or by ripping open the abdominal cuticle. All these methods allowed good penetration of fixatives, but the first is perhaps the easiest and least traumatic. Head capsule incisions were performed with larvae immersed in small drops of 3.5% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.2). Larvae were fixed in glutaraldehyde for 1 hr, washed in cacodylate buffer, and postfixed for 1 hr in 2% OsO₄ in the same buffer. After several rinses in distilled water, larvae were prestained for 4 hr or overnight in 2% aqueous uranyl acetate, dehydrated in an ethanol series, and embedded in Epon. Sections were examined in either a Siemens Elmiskop I or a Hitachi HU 11E electron microscope.

RESULTS

MIV. Unexpectedly, no virus particles (fed unfixed) were at first detected in the lumen of the midgut, and the question arose as to whether virus was in fact being ingested in detectable amounts. This was ascertained by feeding larvae on equivalent samples of doubly fixed MIV prepared from original unfixed inocula. (Fixation in glutaraldehyde alone was ineffective in preserving the integrity of virus particles in the midgut lumen.) In this case, virus particles were found in abundance in the lumen of the gut (Fig. 1). This being so, it seemed reasonable to suppose that unfixed particles were ingested in similar numbers but were destroyed at some point during their passage through the midgut. In order to test this hypothesis, a thorough examination of the more anterior regions and contents of the alimentary canal was undertaken.

The general disposition of epithelia in the anterior regions of the gut is diagrammed in Fig. 2. Intact unfixed MIV virus particles were indeed found in large numbers, but only in the foregut (Fig. 2 and 3) and extreme anterior portion of the midgut (Fig. 4 and 5). In this study, such particles were not found more than approximately 100 μ m distal to the posterior limits of the foregut invagination (Fig. 2A), and no obvious MIV-related structures whatever were seen more than 150 to 180 μ m beyond this point. In region BC, 100 to 180 μ m beyond B, a large number of "empty" virus particles were found (Fig. 6); at higher magnifications, many of these appeared broken (inset, Fig. 6). Fixed granulosis virus capsules, which are not degradable in at least the anterior third of the midgut, were mixed with some preparations of unfixed MIV and used as a marker to show that the distribution of ingested material was

more or less the same throughout the anterior regions of the midgut (Fig. 7 and 8); that is, the presence of capsules 200 to 300 μ m beyond point A would suggest that MIV particles were not restricted in movement down the alimentary canal. This would suggest in turn that the absence of MIV in the more posterior regions was attributable to progressively complete degradation of virions during their passage through the gut.

Virus particles and other ingested materials were rarely seen in the spaces between the anterior region of the midgut and the apposing epithelium of the foregut invagination (Fig. 2-4) and never anterior to the ring valve (Fig. 2 and 3); this space is in any case quite narrow, and the entrance to it often occluded as a result of contact between the peritrophic membrane of the midgut and the cuticular lining of the foregut (asterisk, Fig. 4). In addition, the ceca were apparently free of ingested particulate material (Fig. 4). In these preliminary studies, no MIV particles, either fixed or unfixed, were detected either in, or on the epithelial side of, the peritrophic membrane in either first- or second-instar larvae. A total of 46 first-instar larvae that ingested MIV was examined; of these, 30 had been fed unfixed, and the remainder fixed, virus.

Larvae examined at intervals of less than 24 hr (e.g., 2 hr) after exposure to virus showed a similar degree of virus degradation in the midgut.

Rates of infection with MIV were low, ranging between 5.2 and 19.7%, with a mean of 8.1% (10 trials).

Other viruses. Less detailed observations have been obtained on larvae fed fixed and unfixed TIV, and unfixed occluded granulosis and nuclear polyhedrosis viruses. In the case of TIV, the results were similar to those obtained with MIV (Fig. 9), except that empty TIV particles (not shown) were not seen with the same frequency or ease.

Unfixed granulosis and nuclear polyhedrosis virions appeared largely undamaged in the midgut lumen, although the occluding capsular and polyhedral protein was dissolved completely. All granulosis virus envelopes appeared to be present and intact (Fig. 10), whereas about 50% of nuclear polyhedrosis virus bundles lacked envelopes (Fig. 11). Both types of virus were found to be concentrated in the vicinity of the peritrophic membrane but were never detected in it or on the epithelial side. However, only a few larvae have thus far been examined.

DISCUSSION

It is generally conceded that insect viruses normally gain entry to susceptible hosts via the alimentary canal. However, in only a few cases is anything known about how ingested virus par-

FIG. 1. Appearance of the midgut of a first-instar *Aedes taeniorhynchus* larva allowed to feed continuously overnight on a suspension of fixed MIV particles. The lumen of the gut is filled with virus. P, peritrophic membrane; M, midgut epithelium. In this and the following legends, use of the adjectives "fixed" and "unfixed" refers to the state of virus particles at time of feeding.

FIG. 2. Diagrammatic representation of the anterior gut epithelia in a first-instar *Aedes taeniorhynchus* larva. The foregut epithelium (F) invaginates into the midgut and folds back upon itself, becoming continuous with the midgut epithelium (M) at the point indicated by the asterisk; the peritrophic membrane (P) originates at the same location. The cuticular intima (see Fig. 3) of the foregut is developed into a thick annular ring ("ring valve," RV) located approximately 15 μ m from the point of entry (A) of the ingested food into the lumen of the midgut. The anterior midgut evaginates to form eight ceca (Ca). The distribution of ingested unfixed MIV is indicated by filled and empty hexagons representing, respectively, intact and partially degraded virus particles. Typical areas of the gut represented in some of the succeeding figures are indicated by rectangles and appropriate figure numbers. The gut is further subdivided into regions so as to facilitate an understanding of those areas in which ingested virus and derived virus-related structures are located; the numbers indicate distances in μ m. The figure is not drawn precisely to scale. Intact, ingested MIV is seen in the lumen of the foregut and in region AB of the midgut. Virus shells predominate in region BC. Ant, anterior; post, posterior.

ticles leave the lumen of the gut and enter susceptible cells (6, 16). In addition, virtually nothing is known about the nature or location of barriers to virus invasion that may be inherent in

the administration of virus per os; that such barriers exist seems obvious in view of the fact that several insect and insect-borne viruses are more efficiently transmitted by intrahemocoelic injection (4, 7, 9, 14, 15). The present study is a preliminary attempt to define more clearly, at least in the case of MIV, some host-dependent parameters relating to viral infectivity and at the same time to determine, if possible, the *in vivo* invasion pathway of the virus.

Observations on the fate of ingested unfixed MIV were similar in all of 30 larvae examined: virus particles uniformly underwent extensive degradation in the anterior portion of the midgut lumen. MIV particles appeared to be destroyed so efficiently and, one assumes, rapidly, that little chance of cellular uptake of intact particles seems possible under normal conditions; indeed, MIV entry was not observed in this study. Clearly, much more intensive investigation will be necessary to establish the invasion pathway of this virus. Nevertheless, certain facts seem obvious: cellular uptake of intact MIV, if it occurs, must do so at a very low frequency; in addition, uptake would be expected to occur mainly in the anterior region of the midgut, as intact particles are not found elsewhere.

The observed degradation of MIV particles in the midgut is of some importance. Unless it is assumed that the infectious entity is a subviral component, such as nucleoprotein or nucleic acid released upon disruption of virus shells [an alternative worthy of consideration, as the isolation of infectious African swine fever virus deoxyribonucleic acid has been reported (1); this virus is thought to be structurally related to the iridescent insect viruses (15a)], then degradation must reduce considerably the number of particles capable of initiating an infection; this would be in keeping with the known inefficiency associated with per os transmission of MIV (8, 18). The cause of virus degradation in this case is presently uncertain; ingested bacteria which are concentrated in the anterior midgut and undergo lysis there could contribute to the phenomenon. Experiments are planned to determine the extent of degradation of MIV ingested by axenically reared mosquito larvae. In any case, whether directly or indirectly, conditions in the lumen of the gut undoubtedly represent a first line of defense against MIV infection.

It is of interest that not all ingested viruses undergo degradation in the gut lumen. While the occluding protein of both a granulosis and a nuclear polyhedrosis virus is dissolved, the virions appear to survive intact. TIV particles, on the other hand, are degraded. Our preliminary observations suggest that virus degradation may be

FIG. 3. Unfixed MIV (V) together with fixed marker granulosis virus capsules (G) in the lumen (L) of the foregut. The foregut epithelium (F) is covered with a cuticular intima (I), consisting of a thin electron-dense surface epicuticle and a thicker underlying endocuticle. The ring valve (RV) is a thickening of the endocuticle. The peritrophic membrane (P) lies on the surface of the midgut epithelium (M) and forms a cylindrical envelope which surrounds ingested material after it leaves the foregut. Virus particles, etc., are usually absent from the spaces between apposing mid and foregut epithelia (asterisks). The large arrow points towards the posterior end of the insect.

FIG. 4. Unfixed MIV (V) and ingested bacteria at the point of discharge of ingested material from the foregut to the lumen of the midgut. The peritrophic membrane (P) does not follow the surface of the midgut epithelium (M) into the lumen of a cecum, the entrance to which is indicated by an arrow. Contact between the cuticular intima and the peritrophic membrane of the apposing foregut (F) and midgut epithelia is indicated by the asterisk.

FIG. 5. Unfixed MIV (V) and fixed marker granulosis virus capsules (G) in the lumen of the anterior midgut within 50 μ m of the region illustrated in Fig. 4. Granulosis nucleocapsids are rod-shaped and extremely electron-dense; each is surrounded by an envelope and occluded within an ovo-rectangular protein crystal. P, peritrophic membrane.

FIG. 6. Unfixed MIV (V) approximately 90 μm posterior to region of the gut shown in Fig. 4. The majority of virus particles appears empty, and these are often seen to be broken at higher magnification (inset). The inner viral membrane, normally associated with the nucleoid (15a), is indicated by an arrow (inset).

FIG. 7 and 8. Midgut contents of a larva fed a mixture of unfixed MIV and fixed granulosis virus capsules (G). Shown are representative areas situated approximately 150 (Fig. 7) and 250 (Fig. 8) μm down the midgut. Recognizable empty virus shells (V) are present only in the more anterior region (region BC of Fig. 2).

FIG. 9. Fixed TIV in the lumen of the midgut. P, peritrophic membrane; M, midgut epithelium.

FIG. 10. Unfixed granulosis virus (GV) in the lumen of the midgut. Note that the occluding protein crystals (see Fig. 5, 7, and 8) are not present, having been dissolved in the anterior region of the midgut. The enveloped nucleocapsids appear intact.

FIG. 11. Unfixed nuclear polyhedrosis virus in the lumen of the midgut. The crystalline protein of the polyhedra has dissolved. Some virus bundles lack an envelope (arrows). P, peritrophic membrane.

related to virus structure, rather than to other parameters such as infectivity for the host involved.

In addition to virus degradation in the gut lumen, the present study illustrates dramatically the existence of a second barrier to virus infection, inherent in the structure of the gut itself. The insect gut typically consists of three main regions: foregut, midgut, and hindgut. Of these, the foregut and hindgut are protected by a thick cuticle which is continuous with the exoskeleton of the insect; it is thought highly unlikely that particulate materials or even macromolecules could readily penetrate this layer. The midgut, on the other hand, is protected only by a less compact, generally more fragile layer known as the peritrophic membrane, through which digestive enzymes and the products of digestion presumably can move freely (19); a priori, the midgut would appear to constitute the primary site of initial virus infection per os. The present study nevertheless establishes the peritrophic membrane as a very effective physical barrier to the passage of several different kinds of viruses out of the lumen of the alimentary canal of first- and second-instar mosquito larvae. Unfixed MIV, TIV, granulosis, and nuclear polyhedrosis viruses were all retained by the peritrophic membrane; moreover, fixed MIV and TIV particles which, because they were not subject to degradation, were present in larger amounts in the gut lumen, were also retained.

The peritrophic membrane in *A. taeniorhynchus* larvae originates as a tenuous surface coating on the microvilli of the extreme anterior midgut cells (asterisk, Fig. 2), becoming a much thicker layer separated from the epithelial brush border at the posterior limits of the foregut invagination (*unpublished data*); it is reasonable to assume that the region of the peritrophic membrane closest to its origin would be most susceptible to penetration by virus particles. The most anterior region of the midgut and of the peritrophic membrane is, however, cut off from contact with the particulate contents of the gut lumen by the close apposition of the midgut epithelium and the foregut invagination, especially beyond the annular cuticular thickening ("ring valve") on the outer surface of the foregut. Virus particles are therefore effectively excluded from that region of the midgut where the peritrophic membrane is least thick. The "ring valve," for which no definitive function is as yet known, may in fact act indirectly as a barrier to virus infection in the most anterior regions of the midgut.

If intact MIV particles gain access to or through the midgut epithelium, then it seems most likely that they can do so only where random breaks

might be present in the peritrophic membrane, and only if these breaks occurred somewhere in the region of the midgut between the ring valve and about 150 μm distal to it; intact virus has not been found elsewhere in the midgut lumen. On the other hand, observations on the invasion of granulosis and nuclear polyhedrosis viruses in larvae of two lepidopterous hosts, *T. ni* and *S. frugiperda*, reveal that these viruses readily penetrate into the peritrophic membrane, and are commonly seen on the epithelial side of it (Stoltz, Summers, and Kawanishi, *unpublished data*). Nevertheless, most intact granulosis and nuclear polyhedrosis virus which gain access to the midgut epithelium probably do so not by direct passage through the peritrophic membrane, but by entry through the random discontinuities which are commonly found in the peritrophic membranes of these insects. This is in extreme contrast to the situation in mosquito larvae, in which no breaks have been detected in the peritrophic membrane. Differences in the integrity of this structure, then, may account in part for differences in relative ease of per os transmission of insect viruses in different hosts.

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LITERATURE CITED

1. Adldinger, H. K., S. S. Stone, W. R. Hess, and H. L. Bachrach. 1966. Extraction of infectious deoxyribonucleic acid from African swine fever virus. *Virology* 30:750-752.
2. Bird, F. T. 1961. Transmission of some insect viruses with particular reference to ovarian transmission and its importance in the development of epizootics. *J. Insect Pathol.* 3:352-380.
3. Clark, T. B., W. R. Kellen, and P. T. M. Lum. 1965. A mosquito iridescent virus (MIV) from *Aedes taeniorhynchus*. *J. Invert. Pathol.* 7:519-521.
4. Faust, R. M., and G. E. Cantwell. 1968. Inducement of cytoplasmic polyhedrosis by the intrahemocoelic injection of freed viral particles into the silkworm, *Bombyx mori*. *J. Invert. Pathol.* 11:119-122.
5. Granados, R. R., and D. W. Roberts. 1970. Electron microscopy of a poxlike virus infecting an invertebrate host. *Virology* 40:230-243.
6. Harrap, K. A. 1970. Cell infection by a nuclear polyhedrosis virus. *Virology* 42:311-318.
7. Jutila, J. W., J. E. Henry, R. L. Anacker, and W. R. Brown. 1970. Some properties of a crystalline-array virus (CAV) isolated from the grasshopper *Melanoplus bivittatus* (Say) (Orthoptera: Acrididae). *J. Invert. Pathol.* 15:225-231.
8. Linley, J. R., and H. T. Nielsen. 1968. Transmission of a mosquito iridescent virus in *Aedes taeniorhynchus*. I. Laboratory experiments. *J. Invert. Pathol.* 12:7-16.
9. McLean, D. M. 1955. Multiplication of viruses in mosquitoes following feeding and injection into the body cavity. *Aust. J. Exp. Biol.* 33:53-66.
10. Martignoni, M. E., P. J. Iwai, K. M. Hughes, and R. B. Addison. 1969. A cytoplasmic polyhedrosis of *Hemerocampa pseudotsugata*. *J. Invert. Pathol.* 13:15-18.

11. Matta, J. F. 1970. The characterization of a mosquito iridescent virus. II. Physicochemical characterization. *J. Invert. Pathol.* 16:157-164.
12. Matta, J. F., and R. E. Lowe. 1970. The characterization of a mosquito iridescent virus. I. Biological characteristics, infectivity, and pathology. *J. Invert. Pathol.* 16:38-41.
13. Paschke, J. D., R. E. Lowe, and R. L. Giese. 1968. Bioassay of the nucleopolyhedrosis and granulosis viruses of *Trichoplusia ni*. *J. Invert. Pathol.* 10:327-334.
14. Smith, K. M., G. J. Hills, and C. F. Rivers. 1961. Studies on the cross-inoculation of the *Tipula* iridescent virus. *Virology* 13:233-241.
15. Stairs, G. R. 1965. Dosage-mortality response of *Galleria mellonella* (Linnaeus) to a nuclear-polyhedrosis virus. *J. Invert. Pathol.* 7:5-9.
- 15a. Stoltz, D. B. 1971. The structure of icosahedral cytoplasmic deoxyriboviruses. *J. Ultrastruct. Res.* 37:219-239.
16. Summers, M. D. 1971. Electron microscopic observations on granulosis virus entry, uncoating and replication processes during infection of the midgut cells of *Trichoplusia ni*. *J. Ultrastruct. Res.* 35:606-625.
17. Summers, M. D., and J. D. Paschke. 1970. Alkali-liberated granulosis virus of *Trichoplusia ni*. I. Density gradient purification of virus components and some of their *in vitro* chemical and physical properties. *J. Invert. Pathol.* 16:227-240.
18. Woodard, D. B., and H. C. Chapman. 1968. Laboratory studies with the mosquito iridescent virus (MIV). *J. Invert. Pathol.* 11:296-301.
19. Zhuzhikov, D. P. 1964. Function of the peritrophic membrane in *Musca domestica* L. and *Calliphora erythrocephala* Meig. *J. Insect Physiol.* 10:273-278.