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Parasitoid virus-like particles destroy *Drosophila* cellular immunity

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ABSTRACT **Parasitoid wasps must avoid the destructive effects of the host's cellular defense system in order to exploit the host hemocoel as a suitable environment for their survival. To protect their eggs from encapsulation by *Drosophila melanogaster* blood cells, *Leptopilina heterotoma* females inject a factor that selectively destroys lamellocytes, the type of *Drosophila* blood cell involved in recognition and encapsulation of large foreign objects. Other types of host blood cells, including the phagocytic plasmatocytes, remain functional. This report demonstrates that the destructive factor for lamellocytes is a virus-like particle (VLP) stored in the reservoir of an accessory gland associated with the female wasp reproductive system. We show that VLPs enter *Drosophila* blood cells *in vitro*. VLPs are found free in the cytoplasm of lamellocytes but are confined to phagocytic vesicles of plasmatocytes. As lamellocytes are susceptible to the VLP infection and plasmatocytes are not, we conclude that the mode of VLP entry and its disposition in the cytoplasm determine the fate of the infected host blood cell.**

Eggs of endoparasitic wasps developing within the hemocoel of another insect must avoid the potential harm posed by the cellular defense system of the host. Some parasitoid eggs have surface features or coating materials that provide passive protection against host blood cell reactions (1–6). The blood cells of other hosts are inhibited from encapsulating parasitoid eggs by secretions that female parasitoids deposit along with their eggs in the host hemocoel (7–10). *Leptopilina heterotoma* females utilize an accessory gland factor to disrupt the function of *Drosophila melanogaster* lamellocytes (11). Lamellocytes are discoidal blood cells that layer around a foreign object to form a tightly bound capsule that subsequently melanizes and permanently seals the intruder (12).

Lamellocytes are abundant in the *Drosophila* larval hemocoel when capsules are being formed, such as in larvae of *melanotic tumor* (*tu*) strains; otherwise their frequency is low (13). They are sticky cells that adhere to each other, so clumps of lamellocytes are commonly found in blood cell samples from *tu* larvae. In *tu* larvae parasitized by *L. heterotoma*, lamellocytes lose their adhesivity and change their shape to an elongated, bipolar form (11, 14). These modifications render lamellocytes incapable of forming cellular capsules around foreign bodies.

The *L. heterotoma* factor that interferes with encapsulation was named lamellolysin because its destructive effects are restricted to lamellocytes. *Drosophila* plasmatocytes, which are phagocytic, and crystal cells that function in the melanization of cellular capsules in the hemocoel (15) remain undisturbed in parasitized larvae (11). Lamellolysin is stored in the reservoir of an accessory gland associated with the reproductive system of the female wasp. The fluid contents of this reservoir can be injected into *Drosophila* larvae or added to *Drosophila* blood cell samples *in vitro* to show the destructive effects of lamellolysin on lamellocytes (11, 16, 17). A preliminary examination of the ultrastructure of the reservoir revealed large aggregates of electron-dense materials dispersed in a less dense matrix (11). The purpose of the present study was to identify the component of reservoir fluid that has antilamellocyte activity.

MATERIALS AND METHODS

Parasitoids. The Leiden strain of *L. heterotoma* was bred on the *tu-Sz*^{ts} strain of *D. melanogaster* at 25°C as described (11). Adult wasps were maintained at 18°C on 50% honey solution. Four- to 6-week-old female wasps were rinsed in 95% ethanol followed by three rinses in sterile distilled H_2O and dissected in phosphate-buffered saline (PBS) for *Drosophila* cells (18) to isolate reservoirs. Fluid was released from the reservoirs into PBS by tearing the reservoir walls with fine forceps.

***In Vitro* Assay.** Details of this procedure are given elsewhere (17). Briefly, hemolymph from 15 *D. melanogaster* giant larvae having the genotype *gt w*^{a} *tu-Sz*^{ts}*/y sc gt*^{X11} *v*^{81ix} *tu-Sz*^{ts} (17) was collected in 1.5 ml of Schneider's culture medium (GIBCO) containing 15% fetal calf serum and 0.07% phenylthiourea. Aliquots of the sample (110–125 μl depending on the experiment) were dispensed to Sykes chambers, and 5–20 μl of test material (or PBS in the case of the controls) was added prior to sealing the chambers. The blood cells in the chambers were examined 4 and 16 hr later.

To determine whether lamellolysin is heat sensitive, samples of reservoir fluid were divided into two portions. One portion was heated at 70°C for 30 min and the other was maintained on ice for 30 min. The samples were then bioassayed for lamellolysin activity on cells *in vitro*. Drop dialysis (19) was performed by floating 50-nm Millipore VMWP membrane filters containing reservoir fluid samples on PBS at 4°C for 2–14 hr.

Density Gradient Centrifugation. Reservoir fluid from 75–100 female wasps was layered on preformed 15–60% step gradients of sucrose in PBS or 10–50% Nycodenz gradients (20) in PBS in 0.6-ml centrifuge tubes. The tubes were centrifuged at 114,000 × *g* for 60 min at 8°C in an SW50.1 rotor in a Beckman model L centrifuge. Nine fractions were collected from each gradient by drawing set volumes in sequence from the top of the tube such that the light-scattering band near the middle of the tube was fraction 5. The fractions from sucrose gradients were dialyzed as described above. A 20-μl sample from each gradient fraction was used to test for lamellolysin activity and the remainder of each fraction was saved for electron microscopy.

Electron Microscopy. Samples from the density gradients were spread on electron microscope grids coated with Formvar and carbon. They were fixed in 1.5% phosphate-buffered glutaraldehyde and negatively stained with 2% phosphotungstic acid at pH 7.

To pellet the particulate material in the light-scattering gradient fractions, the samples were centrifuged in an Eppendorf microcentrifuge for 30 min at 4°C. The pellets were

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Abbreviation: VLP, virus-like particle.

fixed in 3.5% glutaraldehyde in 0.2 M sodium cacodylate buffer at pH 6.9. Following postfixation in 1% osmic acid in sodium acetate/veronal buffer at pH 7, the pellets were dehydrated through a graded series of ethanol and embedded in epon. Thin sections were stained with uranyl acetate and lead citrate.

The methods for fixing pellets of gradient fractions and preparing sections of the pellets for transmission electron microscopy were used for samples of *Drosophila* blood cells exposed to reservoir fluid or gradient-purified virus-like particles (VLPs). One experiment was done to determine the time required for VLPs to enter *Drosophila* cells. For this study reservoir fluid was mixed with a sample of *Drosophila* blood cells and an aliquot of cells was fixed immediately (0 min). Two more aliquots of cells were fixed at 10 and 30 min. Sections of the cells were examined in the electron microscope. VLPs were found in the blood cells fixed at 30 min but not in the 0- to 10-min samples. Therefore, 25 min of incubation was selected for the subsequent experiment, which utilized gradient-purified VLPs from 35 female reservoirs. The observations on VLPs in *Drosophila* cells given in this report were made on the cells incubated with gradient-purified VLPs for 25 min.

Female wasps were dissected in PBS to isolate long glands and reservoirs, which were fixed in 3.5% glutaraldehyde and processed for transmission electron microscopy as described above. Grids were examined in a Zeiss EM109.

RESULTS

Characterization of Lamellolysin. Samples of reservoir fluid heated at 70°C for 30 min did not induce elongation of lamellocytes *in vitro*, whereas control samples maintained at 4°C did. Reservoir fluid dialyzed for 14 hr retained lamellolysin activity when tested by the *in vitro* bioassay. Therefore, lamellolysin is a heat-labile, nondialyzable component of reservoir fluid.

Whether lamellolysin is associated with the particulate materials seen in the reservoir lumen by electron microscopy was determined by centrifuging reservoir fluid in density gradients to separate the particulate components. After centrifugation in sucrose gradients, a single light-scattering band was found in the centrifuge tubes. The material in the band and fractions of the gradient above and below the band were separately collected, dialyzed, and added to aliquots of a *Drosophila* blood cell sample *in vitro*. Lamellocytes exposed to all of the gradient fractions retained their discoidal shape.

Viral infectivity has been better preserved in Nycodenz gradients than in sucrose gradients (21). Furthermore, this iodinated density gradient medium is nonionic and nontoxic for cells. Therefore, we tested lamellocyte response to reservoir fluid in the presence of Nycodenz. Nycodenz did not interfere with the changes in lamellocyte morphology induced by lamellolysin nor alter the normal discoidal shape of the control cells, so it is not necessary to dialyze gradient fractions after centrifugation to remove Nycodenz. When reservoir fluid was centrifuged in Nycodenz gradients, a single light-scattering band was present in the centrifuge tubes (Fig. 1*b*). Gradient fractions were collected as previously and tested for lamellolysin activity. Lamellocytes became elongated in the presence of the band material but remained normal when exposed to other gradient fractions (Fig. 1 *a* and *c*).

An aliquot of each Nycodenz gradient fraction spread on an electron microscope grid was negatively stained and examined in the electron microscope. Grids with material from the light-scattering band contained heavy concentrations of VLPs measuring ≈300 nm (Fig. 1*d*). Other gradient fractions were free of particulate material. The entire experiment, including bioassay and electron microscopy of gradient fractions, was repeated two more times with the same results.

Material in the light-scattering band from a sucrose gradient was examined by electron microscopy to compare its contents with those from Nycodenz gradients. The same particles found in Nycodenz fractions were present in the sucrose fraction. Loss of lamellolysin activity following centrifugation in sucrose gradients may have resulted from

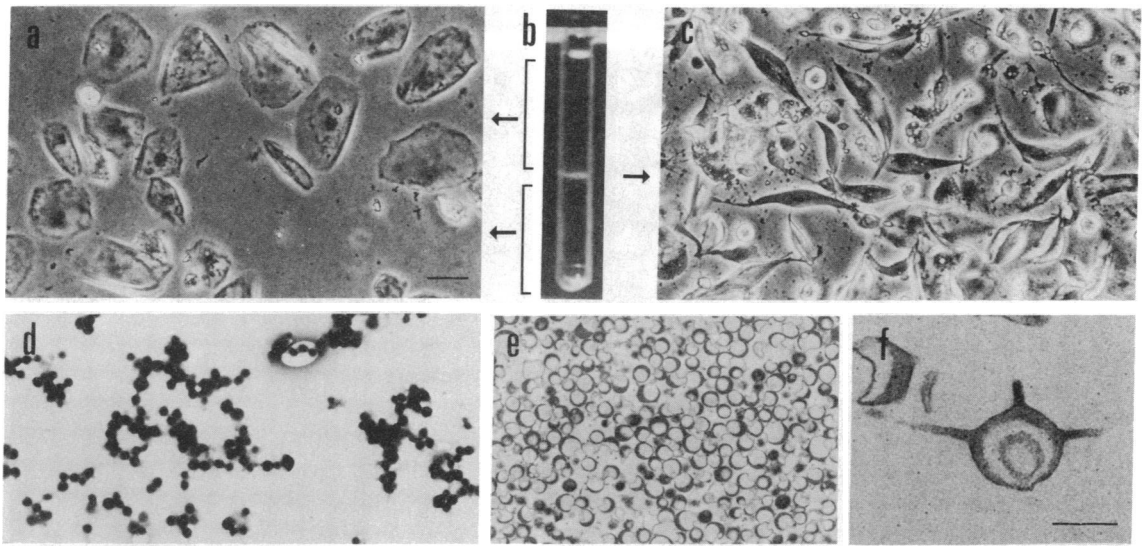

FIG. 1. Isolation and biological activity of the VLPs from *L. heterotoma* reservoir fluid. (*a*) Lamellocytes incubated with Nycodenz gradient fractions 1–4 and 6–9 for 4 hr had normal morphology as illustrated by this photograph of the control cell sample. Some of the discoidal lamellocytes have bright phase contrast at their borders due to folding of their thin edges. (Bar = 50 μm.) (*b*) Centrifuge tube with a Nycodenz gradient that has a light-scattering band at the level of the right arrow near the middle of the tube. Light is also reflected from the curvature at the bottom of the tube and the top of the gradient. (*c*) Lamellocytes treated with the light-scattering band (right arrow, fraction 5) for 4 hr are elongated and show the typical bipolar shape induced by lamellolysin. The small round cells with bright phase contrast are plasmatocytes (same magnification as *a*). (*d*) Electron micrograph of the aliquot from the light-scattering band negatively stained with phosphotungstic acid (×8100). (*e*) Thin section through a pellet of the particles from the light-scattering band. (*f*) Section through a VLP from the sample in *e* showing the surface spikes and involution forming an internal structure. (Bar = 0.22 μm.)

exposure to sucrose or from dialysis required to remove sucrose from the gradient fraction. Lamellolysin activity is not lost by dialysis of reservoir fluid as described above, so an experiment was undertaken to determine whether dialysis following centrifugation results in loss of lamellolysin activity. Material from the light-scattering band from a Nycodenz gradient was dialyzed prior to bioassay. No lamellolysin activity was detected in the dialyzed sample, although dialyzed and nondialyzed samples of the light-scattering band contained VLPs when examined by electron microscopy. These observations suggest centrifugation results in damage to the particles so that essential molecules associated with VLP infectivity are lost upon dialysis.

The particles in the light-scattering band from a Nycodenz gradient were pelleted and sectioned for examination by transmission electron microscopy. In section, the VLPs are spherical with prominent surface spikes extending from electron-dense material, which is asymmetrically distributed in the form of a crescent profile (Fig. 1 *e* and *f*). At high magnification a tightly adhering membrane can be seen surrounding the electron-dense material. Opposite to the widest part of the electron-dense material there is an involuted region forming a vesicle with less electron-dense contents. The particle thus has an internal structure that is also asymmetrically organized.

VLPs in *Drosophila* Blood Cells. Since the gradient fraction having lamellolysin activity was a purified sample of VLPs, it is likely that the VLPs are responsible for lamellocyte destruction. If so, the VLPs must either enter lamellocytes or attach to their surfaces to affect the morphology and function of the cells. As the plasmatocytes are refractory to lamellolysin, it follows that VLP behavior with respect to these cells should differ from that observed in lamellocytes. How VLPs affect lamellocytes was examined by adding gradient-purified VLPs to an *in vitro* blood cell sample and subsequently fixing the cells for transmission electron microscopy.

The elongated profiles of the discoidal lamellocytes can be used to distinguish these cells from the spherical plasmatocytes and crystal cells in ultrathin sections. Crystal cells contain prominent paracrystalline cytoplasmic inclusions that are unique to this blood cell type (13, 22), so sections of these cells are easily recognized. The frequency of crystal cells is low in some *Drosophila* strains and the cells are unusually fragile (23). No cells with paracrystalline inclusions

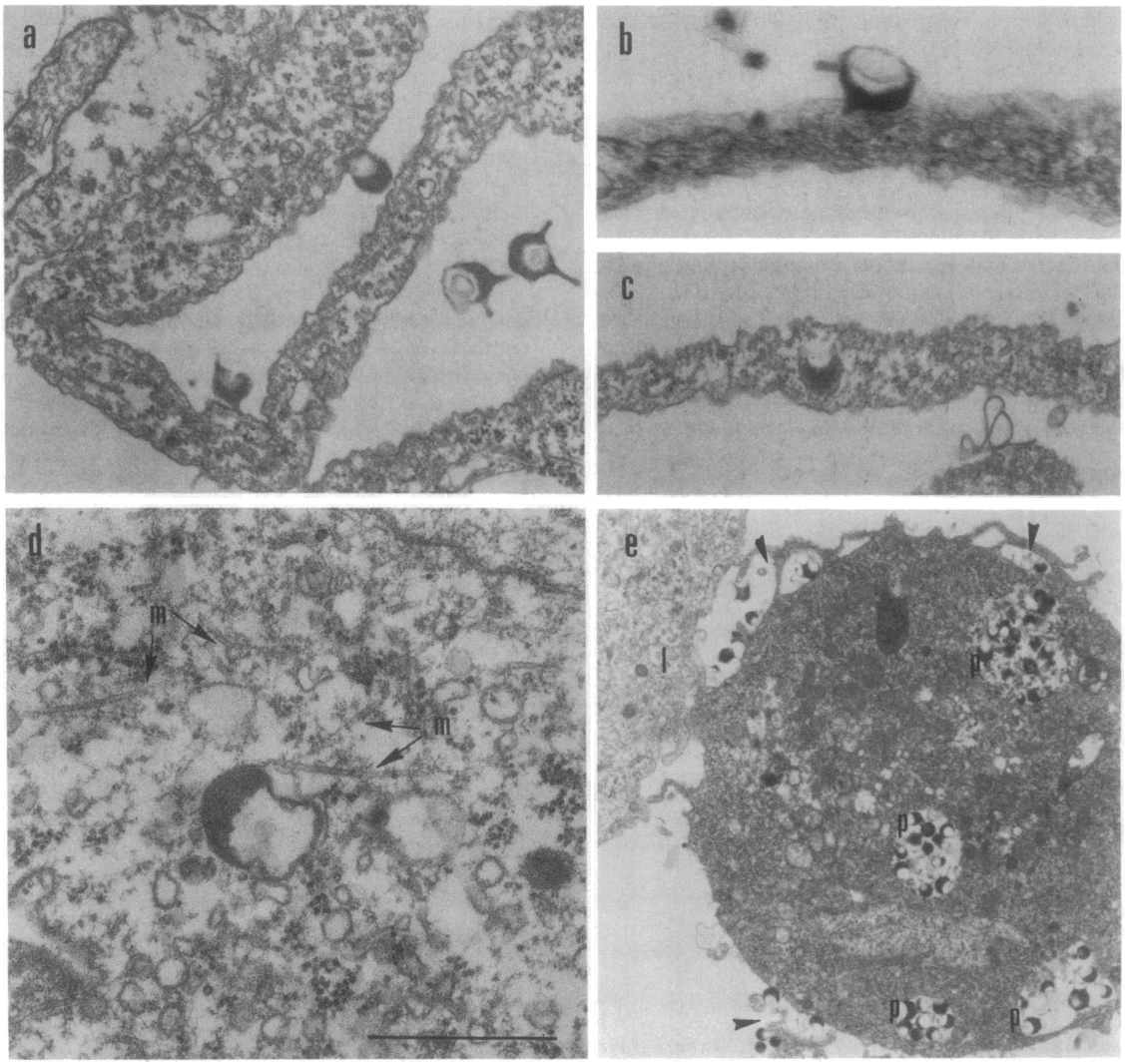

FIG. 2. Electron micrographs of *Drosophila* blood cells treated with gradient-purified VLPs *in vitro*. (*a*) Section passing through the folded, peripheral region of a discoidal lamellocyte showing the attachment of two VLPs to the cell surface. Two free VLPs showing surface projections and internal structures are present at the right side of the micrograph (×21,200). (*b*) A VLP penetrating the surface of a lamellocyte (magnification scale in *d*). (*c*) A VLP in the cytoplasm of a lamellocyte (magnification same as *a*). (*d*) A VLP in a section of a lamellocyte that shows cytoplasmic organelles and microtubules (m). (Bar = 0.5 μm.) (*e*) Section through a plasmatocyte showing phagocytosis of VLPs. Numerous VLPs are contained in four large phagosomes (p) within the cell and other VLPs at the cell surface are being captured in the forming phagocytic vesicles (arrowheads). The surface of a lamellocyte (l) at the upper left corner is free of VLPs. (×9000.)

were found in the blood cell samples examined in the present study. Either the frequency of crystal cells in the *Drosophila* strain used is unusually low or the cells had discharged their paracrystalline inclusions during the *in vitro* incubation that preceded fixation for electron microscopy. Using the criterion of cell shape to distinguish lamellocytes from plasmatocytes, two ultrastructural differences were found between these cell types. Microtubules were abundant in lamellocytes but only a few microtubules were found in plasmatocytes. Also, there was a heavy electron-dense deposit along the nuclear membrane of the lamellocyte that was absent in the plasmatocyte. After establishing that these ultrastructural differences were consistent for the two cell types, these features were also used to identify lamellocytes and plasmatocytes. The electron microscope study surveyed about 600 lamellocyte profiles and all the plasmatocytes in the same epon sections.

The spikes of the VLPs adhere to lamellocyte surfaces and the VLPs enter lamellocytes where they are found free in the cytoplasm (Fig. 2 *a–d*). VLPs in the cytoplasm (Fig. 2 *c* and *d*) often appeared open or more swollen than VLPs outside the cells.

VLPs were also found in plasmatocytes. However, all of the VLPs in these cells were enclosed in phagocytic vesicles (Fig. 2*e*). An additional difference between VLPs associated with plasmatocytes and lamellocytes was also noted. Many VLPs were found near or at plasmatocyte surfaces and in the endocytic vesicles in these cells. Few VLPs appeared at lamellocyte surfaces and few recognizable VLPs were found in the lamellocyte cytoplasm. We also noticed that VLPs generally adhered to the periphery of the lamellocyte where the discoidal cell is thin rather than to the central region of the cell containing the nucleus.

Morphogenesis of the VLP. The identification of lamellolysin as a VLP raises the question why VLPs were not detected in the preliminary ultrastructural examination of the female wasp reservoir mentioned in an earlier publication (11). To answer this question, we undertook a more extensive ultrastructural study of the reservoir and examined thin sections of the long gland associated with the reservoir.

The reservoir is connected to the gland by a single duct as illustrated in the drawing in Fig. 3. The distal region of the gland contains secretory cells and small efferent ducts leading to larger ducts that empty into a main duct that leads to the reservoir. Detailed study of the secretory cells of the gland, duct morphologies, and reservoir structure will be published elsewhere. The present communication focuses on the structure of the VLPs within the gland and the reservoir. The regions of the reservoir and gland in which VLPs were photographed are indicated in the line drawing in Fig. 3.

Cells in the proximal region of the gland contain rows of spherical particles that measure 29 nm and have electron-dense cores (Fig. 3*a*). The structure of these particles and their disposition in the gland are similar to that described for portions of virus-infected cells (24). At the cell borders facing the central duct of the gland, the 29-nm particles enlarge and are associated with asymmetric vesicular units (Fig. 3*b*). In some cases a small electron-dense particle at one side of a large vesicle was identified. It cannot be established from these observations whether the large vesicle is synthesized by the small particle or whether the secretory cells in the distal region

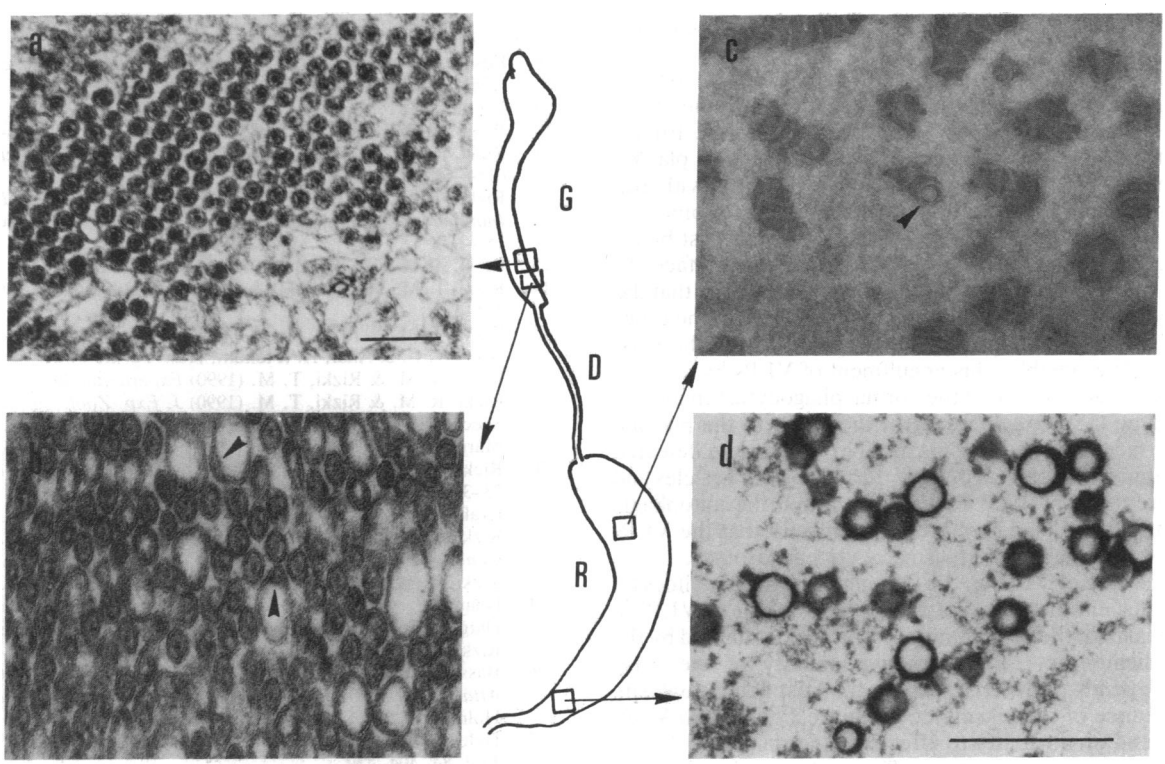

FIG. 3. Morphogenesis of VLPs in the gland and reservoir of the *L. heterotoma* female. The outline of a dissection of a gland (G) and the reservoir (R) connected by a duct (D) shows the origins (squares) of the electron micrographs, *a–d*. The total length of the structures is 1.2 mm. (*a*) Portion of a cell from the proximal region of the gland showing the regular arrays of 29-nm particles with electron-dense cores. (Bar = 0.1 μm.) (*b*) Enlargement and association of the 29-nm particles with large vesicular units (arrowheads) (same magnification as *a*). (*c*) Section through the lumen of the reservoir showing electron-dense aggregates dispersed in a less electron-dense matrix. The circular profile (arrowhead) is asymmetrically electron dense and resembles a section through an asymmetric particle in Fig. 1*e*, suggesting that the aggregates in the reservoir lumen are clumps of VLPs. The discreteness of this object and the one to the upper left was rarely found in the electron-dense aggregates. The adhesion between the VLPs from the gradient in Fig. 1*d* may be due to the native state of the aggregates in the reservoir lumen (magnification same as *d*). (*d*) Mature VLPs from the proximal region of the reservoir resemble gradient-purified VLPs that enter *Drosophila* cells as shown in Fig. 2. (Bar = 1 μm.)

of the gland provide the material of the large vesicle, which subsequently assembles with the small core particle.

The reservoir lumen contains aggregates of electron-dense materials (Fig. 3*c*). These aggregates in the reservoir lumen are the same as observed in the earlier study (11). Presumably, the asymmetric particles from the gland cells aggregate as they are transported to the gland ducts that carry them to the reservoir for storage. Individual particles that resemble the asymmetric particles or the gradient-purified VLPs are not resolvable within the aggregates.

At the proximal end of the reservoir where it connects to the ovipositor, dispersed VLPs that show all of the features of gradient-purified VLPs are found. These features include surface projections and an internal element (Fig. 3*d*). As the morphology of the individual asymmetric unit entering the gland duct for transport to the reservoir differs from that of the mature VLP near the reservoir exit, morphogenesis of the particle must be completed during the aggregation phase.

DISCUSSION

The factor utilized by the *L. heterotoma* female to block the encapsulation response of the *Drosophila* larva is a VLP. VLPs enter *Drosophila* lamellocytes to affect their morphology and surface properties. We presume that the normal discoidal shape of the lamellocyte and the adhesivity of its surface are important for the role these blood cells play in layering around foreign objects to form capsules (25). That lamellocyte shape and surface adhesivity are affected by the VLP infection suggests a relationship between these cell properties. The nature of this relationship is unknown. However, it is clear that the destruction of lamellocyte adhesiveness assures that the cells cannot adhere to form a capsule around a parasitoid egg.

Receptors for viruses on host cells are cell surface components that serve a normal function in the cells rather than molecules synthesized by the cells to bind virus (26). Enveloped viruses enter cells either by fusing with the plasma membrane or by endocytosis followed by fusion with the endosomal membrane after acidification of the endosome (27, 28). The mode of entry of the VLP differs for the host blood cell type. VLPs bind to and penetrate lamellocyte surfaces to enter the cytoplasm of these blood cells. This suggests that the lamellocyte has surface receptors for the VLPs. On the other hand, VLPs are engulfed in phagocytic vesicles by the plasmatocytes. Presumably, this engulfment of VLPs by plasmatocytes is a manifestation of the normal phagocytic function of this cell type (15), so adherence of the VLPs to the plasmatocyte surfaces may be nonspecific. No VLPs were detected in plasmatocyte cytoplasm outside the phagocytic vesicles and there were signs of VLP destruction within the autophagic bodies in these cells. Therefore, it is unlikely that the VLPs escape the confines of the plasmatocyte phagosomes.

The presence of VLPs in the cytoplasm of the lamellocyte, which is susceptible to the VLP, and the absence of VLPs in the cytoplasm of the plasmatocyte, which is unaffected by the VLP, indicate that the destructive activity of the wasp particle depends on its free state in the host cell cytoplasm. The resistance of the plasmatocyte to the *Leptopilina* VLPs must depend on its ability to effectively contain the VLPs in phagosomes and destroy them. These observations suggest that the progression of the host cellular immune deficiency caused by the parasitoid VLP can be modulated by the phagocytic function of the plasmatocyte. As the electron microscope examinations of lamellocytes and plasmatocytes *in vitro* revealed that microtubules are abundant in lamellocytes and not in plasmatocytes, the cytoskeletal differences between the two blood cell types may relate to the cell surface differences between the cells that underlie the specificity of the VLPs for the lamellocytes.

The morphology of the *L. heterotoma* VLP and the complex morphogenesis that this particle undergoes within the accessory organs of the female reproductive system distinguish it from the polydnaviruses (29–31) that are extruded from the calyx cells in the oviducts (32) of ichneumonid and braconid parasitic wasps. Polydnaviruses coat parasitoid eggs as they pass through the oviduct. The suppression of host immune reactions by these viruses (8) may not operate directly on host blood cells (33), whereas the *L. heterotoma* VLP targets host lamellocytes for destruction. Furthermore, the *L. heterotoma* VLP is produced in a separate organ not associated with the oviduct. The reservoir of this gland opens directly into the genital valves. The major question, therefore, is whether the VLP of *L. heterotoma*, a cynipid wasp, is a virus with molecular similarities to the viruses of ichneumonid and braconid parasitoids or whether the cynipid wasp has evolved a defense strategy that employs a different type of infective agent to protect its eggs from harmful effects of host blood cells.

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