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Immunology

Selective destruction of a host blood cell type by a parasitoid wasp

(*Drosophila* hemocytes/cytolytic factor/wasp lamellolysin)

R. M. RIZKI AND T. M. RIZKI

Division of Biological Sciences, The University of Michigan, Ann Arbor, MI 48109

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ABSTRACT **Foreign objects that enter the hemocoel of *Drosophila melanogaster* larvae are encapsulated by one type of blood cell, the lamellocyte, yet eggs of the parasitoid wasp *Leptopilina heterotoma* remain unencapsulated in *D. melanogaster* larval hosts that have many lamellocytes. Here we demonstrate that shortly after a female wasp oviposits in the hemocoel the lamellocytes undergo morphological changes and lose their adhesiveness. These affected blood cells are eventually destroyed as the parasitoid egg continues its development. The factor responsible for lamellocyte destruction, lamellolysin, is contained in an accessory gland of the female reproductive system and is injected along with the egg into the host hemocoel. Lamellolysin does not alter the morphology or the defense functions of the other types of blood cells in the host.**

Large foreign bodies entering the hemocoel of an insect are encapsulated by the circulating blood cells and permanently sealed in melanized cellular capsules. Thus, the successful development of a parasitoid wasp egg in the hemocoel of an insect host depends upon its ability to avoid encapsulation by its host's blood cells. Some parasitoid wasp eggs have special surface features so that they do not arouse a host defense response (1, 2). Other wasps actively interfere with host blood cell function when they oviposit. In the latter category are the ichneumonids that coinfect their hosts with viruses during oviposition so that the host's immune system as well as its growth are influenced (3–5). When melanotic tumor (symbol, *tu*) mutant larvae of *Drosophila melanogaster* are parasitized by some strains of the cynipid wasp, *Leptopilina heterotoma* (formerly, *Pseudeucoila bochei*), the encapsulation of host aberrant tissues to form the inert black masses known as melanotic tumors is blocked together with the inhibition of encapsulation of the wasp eggs (6, 7). The hemocytes of parasitized and unparasitized insect hosts have been compared (8–10), but how the host's immune system is suppressed by parasitoid wasps has not been elucidated.

In this report we describe the loss of adhesiveness and eventual destruction of the type of blood cell that encapsulates foreign bodies. We also demonstrate that the factor responsible for suppression of encapsulation in the host is present in the reservoir associated with an accessory gland of the female wasp reproductive system.

MATERIALS AND METHODS

Insects. A sex-linked, temperature-sensitive melanotic tumor mutant of *D. melanogaster*, $tu(1)Sz^{ts}$, which develops melanotic masses in the posterior fat body when the larvae are grown at 26°C but not at 18°C (11), was used for the experiments. Larvae were raised at 26°C on cream of wheat/molasses medium seeded with live yeast. Two autosomal recessive melanotic tumor mutations, *tu(2)W* and *tu(2)bw* (12, 13), were used to confirm that the effects of parasitization on lamellocytes are not strain specific. For exposure to female wasps, early third instar larvae were rinsed with distilled H_2O and transferred to filter paper moistened with 0.2% glucose solution. They were kept at room temperature with female wasps for 2 hr unless noted otherwise in specific experiments. Following parasitization the larvae were returned to regular food. Control larvae were maintained on glucose solution for 2 hr.

All experiments reported here utilized the Leiden strain of *L. heterotoma*; cursory examination of lamellocytes from *Drosophila* hosts parasitized by two other strains (Mallorca and Storrs) gave similar results. Adult wasps were maintained in vials containing cotton moistened with dilute honey solution. For most of the studies, naive females were used to ensure that a good percentage of larval hosts would be infected. Each *Drosophila* larva was dissected to verify that its hemocoel contained a wasp egg. Under the conditions used, many larvae contained more than one egg; the highest number found was seven. Superparasitism occurs in *Leptopilina* but only one larva successfully continues development so one adult wasp emerges from each *Drosophila* pupal case (14).

Hemocyte Examination. Hemolymph samples were collected in *Drosophila* Ringer solution and examined immediately with phase optics. For the studies with wheat germ agglutinin (WGA) the cells were treated and photographed as described (13). Indirect immunofluorescence studies with anti-tubulin followed published procedures (15–17); for the photographs in this report cells were fixed in 3.7% paraformaldehyde, rinsed in phosphate-buffered saline (P_i/NaCl), and subsesquently permeabilized by treatment with 0.2% Triton X-100 for 2 min. Following P_i/NaCl washes, they were incubated in anti-tubulin (Miles Scientific Code no. 65-095, diluted 1:10 with P_i/NaCl) for 45 min at 37°C and rinsed with P_i/NaCl for 15 min and normal goat serum (diluted 1:20 with P_i/NaCl) for an additional 15 min. They were then incubated in fluorescein isothiocyanate-conjugated anti-rabbit IgG (Miles Scientific Code no. 65-173, diluted 1:16 with P_i/NaCl) for 45 min at 37°C, rinsed in P_i/NaCl, and examined with a Zeiss fluorescence microscope fitted with a dark-field ultracondenser and 40× apochromat objective.

Microinjection Studies. Samples were injected into etherized *Drosophila* larvae by using a hand-held, noncalibrated micropipette from which the fluid contents were dispensed by pressure to a rubber bulb. The amount of fluid injected was gauged by observing a slight distension of the etherized larva. When larvae began moving and showed signs of wound healing they were returned to containers with food.

For the studies on phagocytosis of bacteria, *Escherichia coli* strain J5 (NIH no. 0111, acquired in 1980) were grown in yeast tryptone broth with glucose. Following several washes in saline, they were suspended in *Drosophila* Ringer solution for injection into etherized larvae. Injected larvae were fixed in formaldehyde and processed for transmission electron microscopy as described (18).

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Abbreviation: WGA, wheat germ agglutinin.

Female wasps were dissected in *Drosophila* Ringer solution and individual organs of the reproductive system were transferred to a small drop (0.5 μl) of fresh Ringer solution and macerated.

RESULTS

The types of blood cells in *D. melanogaster* larvae have been described (19). Briefly, these include two classes of spherical cells, crystal cells and plasmatocytes. Crystal cells contain phenol oxidases required for melanization of cellular capsules and wound healing (20, 21). Plasmatocytes are phagocytic cells (18) that also differentiate into highly flattened, nonphagocytic cells, the lamellocytes (22). Lamellocytes form the laminated walls of melanotic capsules in *tu* mutants during the third larval instar. Therefore, third instar *tu* larvae have many hemocytes potentially capable of encapsulating foreign materials placed in the hemocoel.

Effect of Parasitization on Lamellocytes. Hemocytes taken from early third instar *tu-Sz*^{ts} larvae 1–2 hr after exposure to wasp females were compared with hemocytes from control larvae. There was no detectable difference in morphology of plasmatocytes and crystal cells in the parasitized and unparasitized larvae. However, the lamellocytes from parasitized larvae were bipolar or elongated rather than discoidal as in control larvae (12, 19). Spikes extended from the stretched tips of some of the elongated lamellocytes and small blebs were often present at these tips as if materials were budding from the cells. Elongated lamellocytes were found in hosts with a single wasp egg as well as hosts containing more than one wasp egg. They also appeared in *tu-W* and *tu-bw* larvae that had been parasitized by *L. heterotoma*.

Equally as striking as the morphological modification of the lamellocytes was the lack of adherence between the elongated cells. Lamellocytes in *tu* larvae adhere to each other and aggregates of these cells are often found in hemolymph samples (Fig. 1*a*). We did not observe any clumping or stickiness among the lamellocytes of infected larvae (Fig. 1*b*). Thus, it appears that the ability of the lamellocytes to adhere to each other, a property essential for layering in capsule formation, is lost when a female *Leptopilina* deposits an egg in the hemocoel of a *Drosophila* larva.

If fragmentation or blebbing at the tips of the elongated lamellocytes takes place, then extruded material should be present in the hemolymph of parasitized larvae. To test this hypothesis, individual larvae were opened under a small drop of mineral oil rather than in Ringer solution so that all contents of the hemolymph sample remained in a confined area. Examination of these samples with phase optics showed numerous small, dark-contrasting droplets (Fig. 1*c*) but no such components were found in hemolymph samples from control larvae examined under mineral oil.

The fate of the affected lamellocytes was determined by sequential sampling of hemocytes from control larvae and larvae that were exposed to wasps at 44 hr of age. After parasitization both groups of larvae were maintained at 24°C to slow development so that two subsequent samples could be taken before pupariation. The results are summarized in Table 1. The percent lamellocytes in parasitized and unparasitized larvae was initially similar (≈34% at 47 hr); however, the bulk of lamellocytes in the parasitized larvae was elongated cells. In the parasitized larvae shortly before pupariation (95 hr) elongated lamellocytes had decreased to 0.3%. Since this decline in elongated lamellocytes was accompanied by masses of cellular debris, we conclude that the affected lamellocytes are eventually destroyed.

There was a low percentage of discoidal lamellocytes in all infected larvae. Several possibilities may account for the presence of these cells: (*i*) there may be a subpopulation of lamellocytes resistant to the infection; (*ii*) the lamellocytes in transition from the plasmatocytic to the lamellocytic form (22) may not be affected by parasitization; (*iii*) the wasp factor that affects lamellocytes may be unstable or depleted by irreversible binding to the affected lamellocyte surfaces since the proportion of discoidal lamellocytes increased with time elapsed from infection (Table 1).

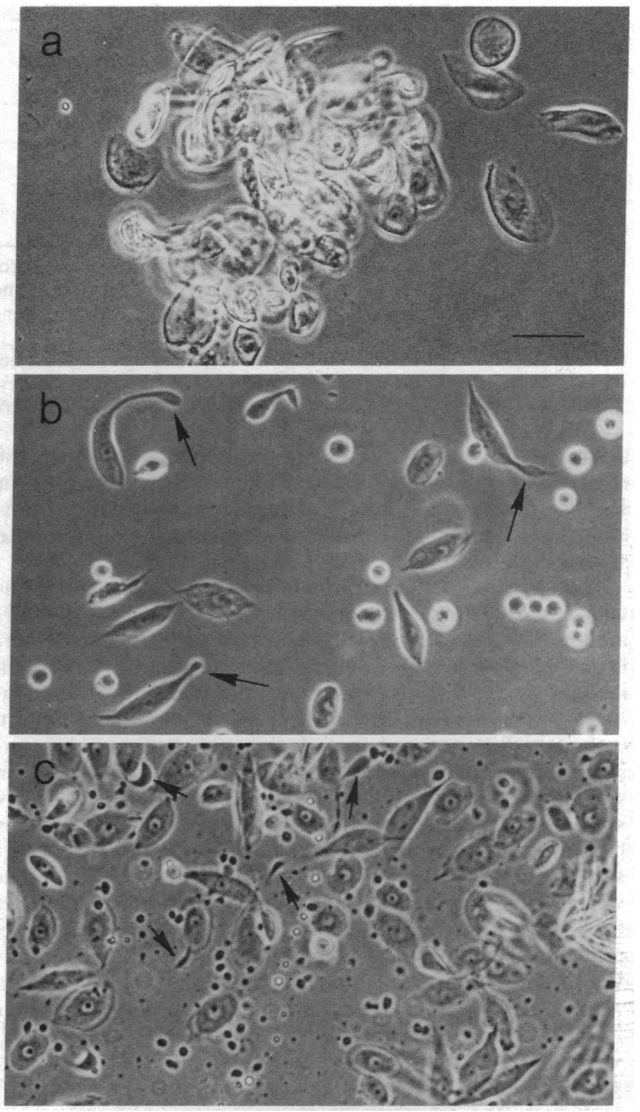


FIG. 1. Phase micrographs of lamellocytes from *tu-Sz*^{ts} larvae raised at 26°C; *a* and *b* in *Drosophila* Ringer solution. (Scale = 40 μm.) (*a*) Discoidal lamellocytes from a control larva (age 47 ± 1 hr) showing the adherence of the cells in clumps. (*b*) Elongated lamellocytes from a larva containing three *Leptopilina* eggs. At 45 ± 1 hr after eclosion the larva was exposed to wasp females for 2 hr at room temperature and its hemolymph was sampled 1.5 hr later. The bipolar cells no longer adhere to each other. Note the budding from the tips of some cells (arrows). For additional comparison of lamellocyte shapes, see Fig. 2. (*c*) Hemolymph sample from a parasitized larva under mineral oil. The sample is full of cytoplasmic blebs extruded from the lamellocytes as they assume the bipolar profiles; some (arrows) are like the tips of the cells indicated in *b*.

The time required for detectable changes in lamellocyte shape following oviposition by a wasp was determined by placing *tu-Sz*^{ts} larvae with female wasps for 20 min and sampling hemolymph from a larva every 5 min beginning 20 min after the wasps and larvae had been separated. In this experiment one-fourth of the larvae had a single wasp egg, one larva had two eggs, and the remaining larvae did not have an egg. The hemolymph samples were classified according to

Table 1. Hemocytes of parasitized and unparasitized *tu-Sz*^{ts} larvae

Host	Age, hr	Plasmatocyte and crystal cell	Lamellocyte				
			Discoidal		Elongated		Discoidal/elongated
Unparasitized	47	2201	1144	34.2%			
	71	9190	3079*	25.1%			
Parasitized	47	6231	64	0.7%	3203	33.7%	0.02
	71	4663	173	2.6%	1762	26.7%	0.10
	95	8553	176	2.0%	26	0.3%	6.77

Hemocytes in each age group are pooled from three larvae. The hemocytes from the first pair of lymph glands had been released into the hemocoel in all larvae. By 95 hr most of the larvae had pupariated and only those larvae preparing for pupariation were sampled. The % values indicate the percentage of the total hemocytes.

*Lamellocytes were encapsulating caudal fat body at this stage so the lamellocyte count is an underestimate. No attempt was made to count hemocytes from 95-hr unparasitized larvae since capsules had formed; clumps of discoidal lamellocytes were present in the hemolymph of these larvae.

the shape of the lamellocytes and the presence or absence of lamellocyte clumps. Elongated lamellocytes appeared in samples with eggs at 90 ± 10 min. None of the larvae containing a wasp egg had cell clumps, a feature common to the samples without eggs.

The adhesive surfaces of lamellocytes can be experimentally modified by dietary glucosamine to affect capsule formation (23). Carbohydrate moieties of the lamellocyte surfaces bind WGA (13). This lectin conjugated to fluorescein isothiocyanate distinguishes two subpopulations of lamellocytes, those whose surfaces light up in a speckled pattern (spk$^+$ lamellocytes) and those lacking this surface characteristic (spk$^-$ lamellocytes). Most lamellocytes in larvae in which capsule formation is in progress—e.g., *tu* larvae—are spk$^+$, whereas lamellocytes of larvae that are not forming capsules are primarily spk$^-$. To determine whether the spk$^+$ surface is modified by *Leptopilina* infection, *tu-Sz*^{ts} larvae were exposed to female wasps for 2 hr and the lamellocytes from these larvae were subsequently treated with WGA. Hemolymph samples were taken from larvae at the time the wasps and larvae were separated, and at 4 and 24 hr later. About 85% of the lamellocytes in hemolymph samples taken from larve at the time the wasps were removed from the vials were spk$^+$. In parasitized larvae the lamellocytes were elongated, whereas in unparasitized larvae they were discoidal. However, the intensity of fluorescence appeared similar regardless of cell shape. At 4 hr after infection the percentage of spk$^+$ elongated lamellocytes in infected larvae was reduced to 66% and many of the cells that were still spk$^+$ were not as strongly fluorescent as in the early samples. By 24 hr after infection only 24% of the lamellocytes in infected larvae had spk$^+$ surfaces, confirming that the surface properties of the lamellocytes are altered by the infection (Fig. 2 *a* and *b*).

Cell shape depends on the disposition of cytoskeletal elements, including microtubules, microfilaments, and intermediate filaments. Whether the transition of lamellocyte shape from discoidal to an elongated form involves rearrangement of the cytoskeleton was examined by using anti-tubulin to visualize the microtubules by indirect immunofluorescence. The distribution of microtubules in control and affected lamellocytes is shown in Fig. 2 *c–e*. Microtubule arrays in lamellocytes from control larvae were heavily concentrated around the nucleus and radiated to the periphery of the cell. The majority of the microtubules in the lamellocytes of wasp-infected larvae was aligned with the long axis of the cells and appeared somewhat fuzzier than those in normal lamellocytes. The latter difference was independent of modifications in the fixation procedure, including Triton X-100, methanol, ethanol, or acetone treatment to permeabilize the cells after formaldehyde fixation. Fluorescent material extended into the elongated tips of the lamellocytes and, in several instances, was present in the form of knobs, indicating the extrusion of tubulin or microtubules (or both) from the affected cells. Thus, the modification of lamellocyte mor-

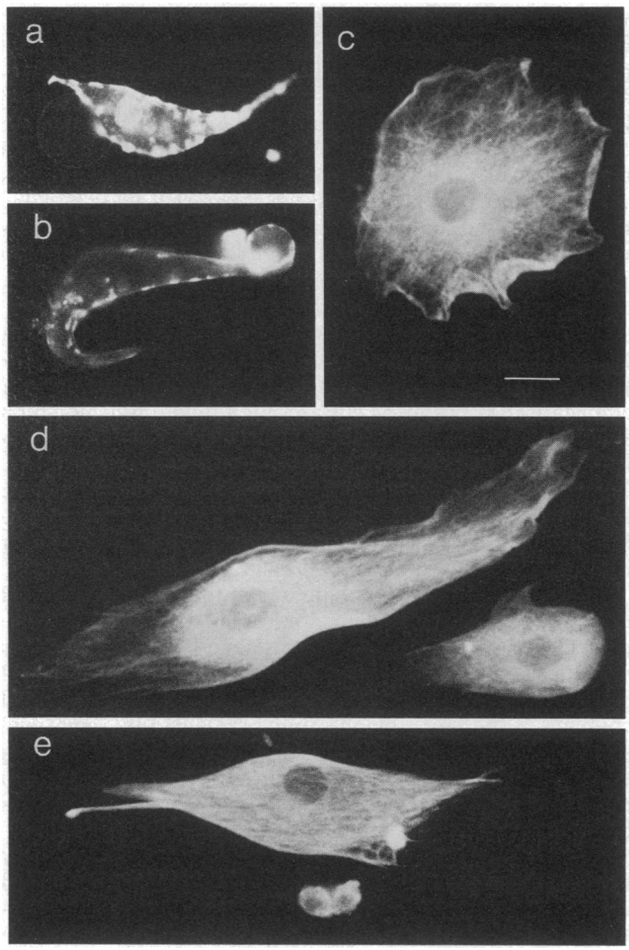


FIG. 2. (*a* and *b*) Elongated lamellocytes from parasitized larvae treated with fluorescein isothiocyanate-WGA. (*a*) spk$^+$ cell at the time the wasps were removed from the vials. The speckling is intense and knob-like, resembling that of normal spk$^+$ lamellocytes (13). (*b*) spk$^+$ cell 24 hr after infection. The speckles are flat patches and less intense than in the earlier sample. The bleb on the right also shows the spk$^+$ characteristic. (*c–e*) Indirect immunofluorescence from anti-tubulin binding to microtubules of lamellocytes. (Scale = 10 μm.) (*c*) Lamellocyte from a control larva. The thinness of the cell can be judged by the folds at the periphery, a feature that cannot be easily visualized with phase-contrast optics as in Fig. 1*a*. The shape of these cells is studied best by scanning electron microscopy (11, 12). (*d* and *e*) Elongated lamellocytes from parasitized larvae. In *e*, tubulin-containing blebs, one at the end of the spike on the left and one at the lower right border of the cell, are apparent.

phology includes changes in the anchorage of microtubules and sloughing of these components together with the lectin-binding surface components.

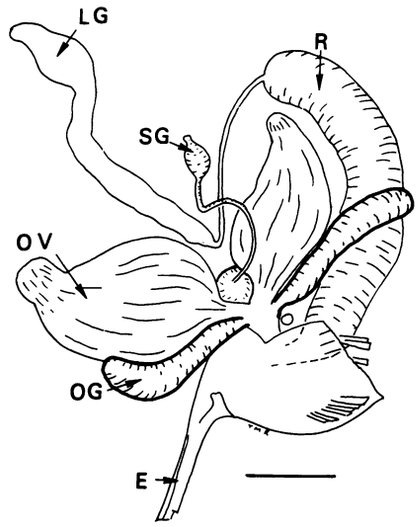
Normality of Plasmatocytes and Crystal Cells. The morphology of the plasmatocytes and crystal cells in wasp-infected larvae was not different from that in control larvae. Either these cells are not so rapidly affected by *Leptopilina* infection as are the lamellocytes or the morphological effects on these cells are not apparent at the level of examination used. We therefore sought to determine whether the defense functions of the plasmatocytes and crystal cells are disrupted by *Leptopilina* infection.

Plasmatocytes are phagocytic cells and this function can be readily seen when bacteria are injected into the hemocoel. To determine whether phagocytosis is interrupted within the same interval that lamellocytes are affected, *E. coli* were injected into *tu-Sz*^{ts} larvae 70–80 min after a 2-hr exposure to wasp females. A group of larvae that had not been exposed to wasps was also injected with bacteria. The larvae were fixed 1 hr after they had received the bacterial injections, and regions of the body walls that form the posterior hemocoel were processed for transmission electron microscopy. Sections through hemocytes in the caudal hemocoel were examined. Bacteria were present in plasmatocytes of both groups of larvae but no bacteria were found in lamellocytes of either sample. We conclude that the ability of the plasmatocytes to phagocytose foreign materials is not disrupted within the interval in which lamellocyte morphology is affected.

That infection with *Leptopilina* does not alter crystal cell function necessary for wound healing was apparent in this same experiment. Wound healing at the injection site, evident as a black scab, was the same in infected and uninfected larvae.

Source of Lamellolysin in Female Wasps. The above studies suggest that oviposition by a female wasp rapidly incapacitates the lamellocytes without apparent influence on the defense functions of the other hemocytes. We therefore propose naming the factor responsible for selective destruction of the lamellocytes, lamellolysin, and in the final group of experiments sought to locate the source of lamellolysin. Among the possible sources are the egg itself or some other material that is injected along with the egg in the host hemocoel by the female wasp. Fig. 3 includes a camera lucida drawing of the ovaries and oviduct of a female *L. heterotoma* together with the accessory glandular structures, labeled long gland with its reservoir, oviducal gland, and sac gland. Each of these organs from a single female was removed and macerated separately in Ringer solution. When the long gland reservoir is squeezed or torn, a whitish fluid is released into the Ringer solution; this fluid was used as the test material for the long gland. Each sample was injected into an etherized host larvae (age 68 ± 2 hr at 26°C). The reproductive organs of two additional wasp females were separated and used for injection in a similar manner. Hemocytes from the host larvae were examined 3.5 hr after injection. Two of the three hosts injected with material from the long gland reservoir has elongated lamellocytes, whereas the lamellocytes from hosts injected with other tissues were normal.

Two subsequent experiments were performed, but in these only the contents of the long gland reservoir and oviducal gland material were injected into larval hosts. Gland material from a single female was used to inject two or three larvae. None of the 15 hosts injected with material of the oviducal gland had elongated lamellocytes. Hemocyte samples from 13 of 16 hosts injected with long gland contents contained elongated lamellocytes (Fig. 3), so we conclude that the long gland is the source of lamellolysin. The lack of response in some cases may be due to uncontrolled variables. For instance, the amount of fluid injected into each larva was not quantified. Also, loss of fluid from the site of the needle puncture varies among injected specimens depending upon postinjection contraction of the larva and the length of time before the wound heals.



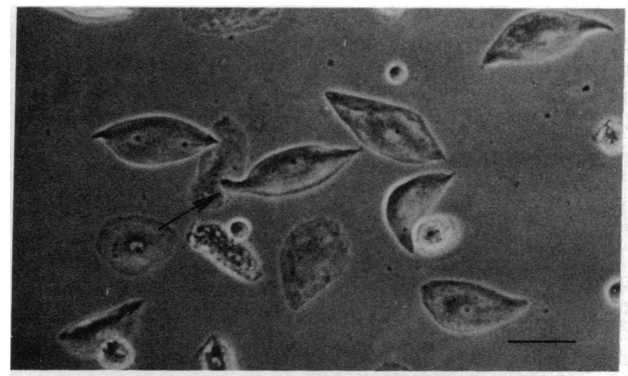


FIG. 3. A camera lucida drawing of the ovary and associated glands of *L. heterotoma*. The gooseneck-shaped long gland, LG, is connected by a duct to its reservoir, R; OG, oviducal gland opening at the proximal end of the oviduct; OV, ovary; SG, sac gland connected by a duct to a reservoir, part of which can be seen between the oviducts; E, portion of the external genitalia. (Scale = 200 μm.) The photograph shows hemocytes from a *tu-Sz*^{ts} larva injected with the contents of the long gland reservoir. Most of the lamellocytes have assumed an elongated shape similar to that found in wasp-infected larvae, although a few discoidal lamellocytes are still present. Note the blunt pole (arrow) forming a knob. Lamellocytes from older larvae are slightly larger than those from younger third instar larvae. The small round cells with strong phase halos are plasmatocytes. (Scale = 40 μm.)

DISCUSSION

The unusually thin, flattened shape of the lamellocytes fits their role in encapsulation. These discoidal cells layer on top of one another to form a capsule around foreign objects (24, 25). Under capsule-forming conditions, such as those found in *tu* mutants, most lamellocytes are capsule competent (13). Therefore, the lamellocytes in *tu* larvae present an immediate danger to a wasp egg in the hemocoel and the survival of the egg depends upon countering this threat. This is accomplished by lamellolysin injected along with the egg into the host hemocoel. The apparent selectivity of lamellolysin may be due to the terminally differentiated state of the lamellocytes since such cells may not be capable of replenishing surface molecules that bind irreversibly to lamellolysin. Whether this is the case and how the other types of blood

cells avoid the destructive effects of lamellolysin remain to be examined.

The nature of lamellolysin also requires investigation. Viruses and virus-like filaments in the calyx of parasitoid wasps have been implicated in suppression of host immunity (4, 10). The relative position of the secretory epithelium of the calyx in the oviduct of these wasps corresponds to the position of the oviducal glands of *L. heterotoma*. However, extracts of oviducal glands had no effect on lamellocyte morphology. Preliminary examination (unpublished observations) of the ultrastructure of the long gland reservoir failed to show particulate material resembling the viruses in the calyx of ichneumonids (4) or the virus-like filaments in the braconids (10). These findings have limited value; they do not exclude the possibility that lamellolysin is the product of a virus sequestered elsewhere in the female wasp. On the other hand, Walker's (6) genetic studies with resistant and susceptible strains of *L. heterotoma* provide information on the nature of the factor that affects host response. When females from lines resistant to encapsulation were mated with males from susceptible lines, and vice versa, host response to the hybrid eggs remained the same as that of the maternal line. Resistance and susceptibility were not influenced by the male parent. Unfertilized eggs (F_2) from both groups of hybrid females were equally susceptible to encapsulation and the encapsulation values were intermediate between those of the two parental lines. Walker (6) concluded that the maternal genotype is responsible for the production of an inhibitor (*I*) of host response, and the resistant lines carry semi-dominant genes for *I*. We assume that lamellolysin and *I* are the same and a product of the maternal genotype.

It is interesting to consider that the destruction of lamellocytes is crucial for protection of the egg while the maintenance of larval health and vitality are also important to the developing parasitoid. In these studies we did not detect a difference between the length of larval life of parasitized and unparasitized hosts. Both groups of larvae appeared equal in size. Nor did we notice any abnormalities in host larval tissues through pupariation; the imaginal disks of the parasitized hosts showed normal eversion at pupation. Obviously it is advantageous to the parasitoid that its host grow and form a normal puparium; the host tissues serve as nourishment for the parasitoid larva and the host puparium as its protective shell. Maintenance of the host cellular defense system, other than encapsulation, guards the host and its harbored parasitoid from incidental infection and injury.

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