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Hemocyte Responses to Implanted Tissues in *Drosophila melanogaster* Larvae

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Summary. At 26° C temperature-sensitive *tu(1)Sz*^{ts} larvae of *Drosophila melanogaster* develop melanotic tumors consisting of aberrant caudal adipose tissue encapsulated by precociously differentiated hemocytes (lamellocytes). When *tu-Sz*^{ts} larvae are grown at 18° C, lamellocytes are present but the caudal fat body surfaces remain normal and melanotic tumors do not develop (Rizki and Rizki, preceding paper). In this paper we demonstrate that the lamellocytes in *tu-Sz*^{ts} larvae at 18° C encapsulate implants of mechanically-damaged fat bodies and adipose cells devoid of basement membrane, while leaving host fat bodies or implanted fat bodies with intact basement membrane unencapsulated. Therefore, low temperature blocks melanotic tumor formation by normalizing the surfaces of the prospective tumor-forming sites in *tu-Sz*^{ts}.

The discriminatory ability of *tu-Sz*^{ts} lamellocytes was examined by challenging them with undamaged heterospecific tissues. Tissues from sibling species of *D. melanogaster* were not encapsulated whereas tissues from *Drosophila* species outside the *D. melanogaster* species subgroup were. Ultrastructural examination of encapsulated heterospecific tissues showed intact basement membrane, so we propose that distinction between "self" and "not self" by lamellocytes depends upon the molecular architecture of the basement membrane. In similar series of experiments using *D. virilis* donor tissues in *Ore-R* wild type larval hosts, fat bodies remained unencapsulated and imaginal disks metamorphosed. These studies suggest that continued presence of lamellocytes in the larval host is a prerequisite for encapsulation.

Key words: Hemocytes – Transplantation – Cell-mediated Defense – Basement membrane.

Introduction

It is generally accepted that insects lack an adaptive immune system and immunoglobulin-like proteins (Salt 1970). They rely primarily on the cell-mediated responses of phagocytosis and encapsulation to combat foreign materials in the hemocoel. Small foreign particles such as bacteria are engulfed by phagocytic hemocytes whereas larger objects, including inanimate materials, parasites and heterospecific tissues, are encapsulated within hemocytic nodules. In melanotic tumor mutants of *Drosophila melanogaster* these same responses are evoked by the body's own tissues which show surface abnormalities during larval development (Rizki and Rizki 1979). Melanotic tumor mutants therefore provide an opportunity to investigate genetic control of blood cell defense responses in an insect amenable to genetic manipulations.

A useful *Drosophila* mutant with which to begin experimental study of the encapsulation reaction is the sex-linked, temperature-sensitive mutant *tu(1)Sz*^{ts} (hereafter abbreviated *ts*). When *ts* larvae are cultured at 26° C they develop melanotic tumors in their posterior fat bodies (Rizki and Rizki 1978). Included in the development of this mutant phenotype is the precocious differentiation of the spherical plasmatocytes to the flattened lamellocytic form, and phagocytosis and encapsulation of aberrant posterior fat body cells by the lamellocytes. When *ts* larvae are raised at 18° C, lamellocytes are present in the hemocoel, the posterior fat body cells remain normal in appearance, and melanotic tumors do not develop. These observations suggest *ts* lamellocytes at 18° C may be competent for encapsulation and the absence of an appropriate site for their activity accounts for the lack of melanotic tumors (Rizki and Rizki, preceding paper). In this report we summarize experiments testing this hypothesis, evaluate the discriminatory ability of the *ts* lamellocytes, and examine the hemocyte responses of *Ore-R* wild type larvae that do not show the precocious presence of lamellocytes.

Materials and Methods

Information on the *tu(1)Sz*^{ts} mutant is included in the preceding paper (Rizki and Rizki 1980). For implantation studies species

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available in our laboratory were used: *D. melanogaster* (Ore-R-CH), *D. virilis, D. willistoni, D. nebulosa, D. novamexicana, D. pseudoobscura,* and *D. simulans* (c). Sibling species of *D. melanogaster* were obtained from Dr. R.C. Woodruff of Bowling Green State University: *D. yakuba* (115), *D. teissieri* (128.2), *D. mauritania, D. erecta* (154.1), and *D. simulans* (c135.20).

Host larvae were grown on cream of wheat medium with live yeast at 18° C. They received tissue implants when they were 5 days old (mid third instar) or approximately 48 h before pupariation under these culture conditions. The first implantation experiments were conducted at 18° C; after determining that exposure of *ts* hosts to room temperature (23°–24° C) for the experimental period (1 h) did not cause tumor formation of the host fat body or produce results different from those experiments conducted at 18° C, implantations were carried out at room temperature and the hosts promptly returned to 18° C. Specimens for donor tissues were grown on cream of wheat medium at either 18° C or 26° C, and donors selected on the basis of the size of tissue required for a given experiment; fat body tissues slightly smaller than those of host larvae were used and imaginal disks were obtained from late third instar larvae.

Host and donor larvae were washed in 1.5% NaClO solution and thoroughly rinsed with distilled H_2O. Tissues were dissected and rinsed with several changes of Ringer solution before they were implanted into host larvae using a glass micropipette. Each host larva which had been lightly etherized received a single implant. Implants were recovered by opening hosts in Ringer solution. Generally, implants were recovered after 48 h or at pupariation but several experiments involved shorter or longer time spans. Some implanted tissues were examined with phase optics immediately upon recovery from the hosts; others were fixed for transmission and scanning electron microscopy, or histological examination.

To inflict mechanical injury to the surfaces of tissue implants, the tissues were gouged several times with the tips of fine forceps. This treatment was sufficient to disrupt the tissue surfaces without breaking the tissues into smaller pieces prior to implantation. For digestion of basement membrane fat bodies were incubated in collagenase (Worthington, CLSPA; 1 mg/ml Ringer for 15 min) and rinsed five times with Ringer solution to remove traces of collagenase. Extreme care was required during this rinsing to maintain the integrity of the tissues which had been weakened by collagenase digestion of the basement membranes. Upon suction into the injection needle and implantation into the host, the fat body cells dispersed singly or in small clusters. Control series of fat body implants for the collagenase experiments were kept in Ringer solution for the same duration of time as the enzyme-treated tissues and then rinsed with Ringer solution prior to implantation.

Results

D. melanogaster (Ore-R) Fat Body Implants in ts Hosts

Since encapsulation of fat body by lamellocytes in *tu-W* and *ts* larvae at 26° C occurs after tissue surfaces are visibly modified (Rizki and Rizki, preceding paper), it is reasonable to expect that implanted fat bodies with modified surfaces should arouse the encapsulation response of the *ts* lamellocytes at 18° C if the latter are capable of encapsulation. On the other hand, fat body tissues that retain a normal surface following implantation should not be encapsulated

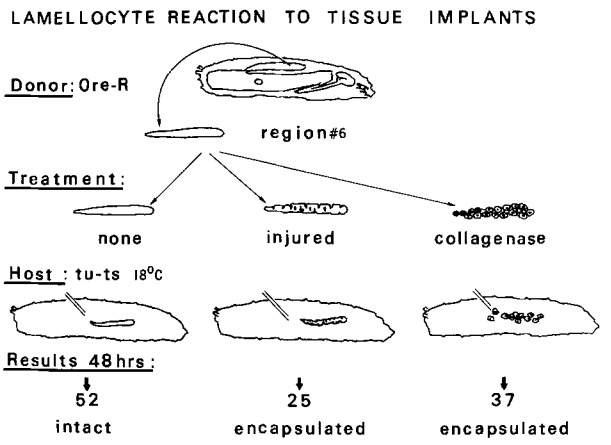
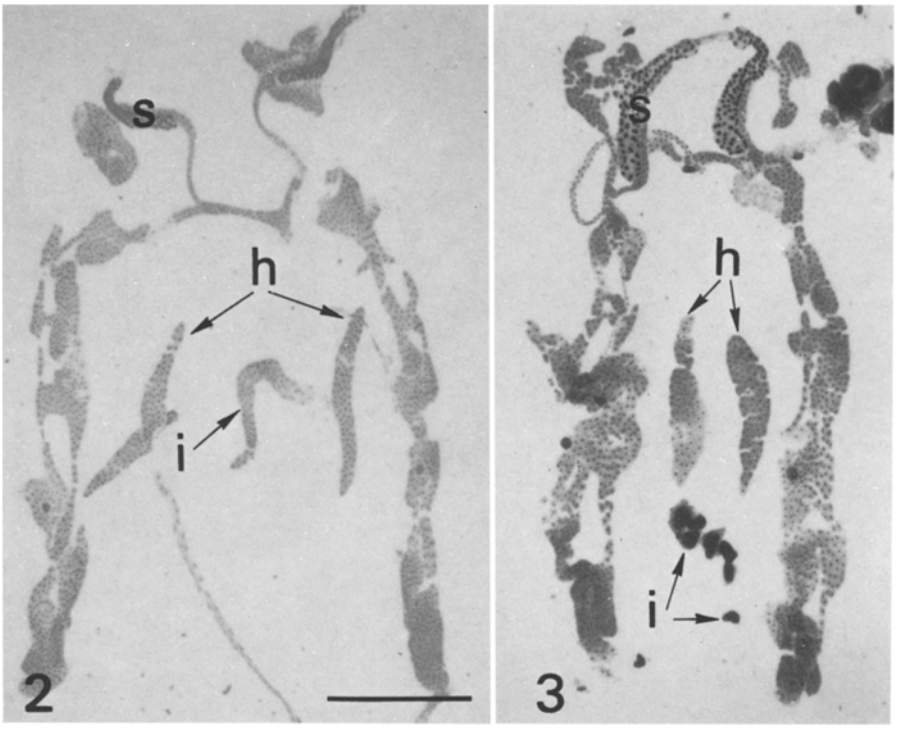


Fig. 1. Design of experiments to test the *ts* lamellocyte response to *Ore-R* wild type donor fat bodies implanted as intact (none) or surface-modified (mechanically injured or collagenase treated) pieces. The summarized results indicate the numbers of implants and their condition at recovery

if the lamellocytes remain neutral to normal tissue surfaces. To determine whether lamellocytes of *ts* can discriminate these two categories of implanted fat bodies it is important to select donor fat body that can be manipulated and implanted without injury to its surfaces. In addition, the pieces of tissue should be of sufficient size to allow experimental modifications to their surfaces prior to implantation and be distinguishable from host tissues at recovery. These requirements are met by the two elongated dorsal regions of the larval fat body designated region "6" (for a description of the larval fat body regions, see Rizki 1964). Region 6s are loosely connected to the main lateral fat masses by single cells at a narrow neck with overlying acellular matrix so they can be easily isolated for implantation without damage to the bulk of the tissue surface. The elongate shape of these large pieces facilitates implanting them into host larvae using small bore micropipettes and is useful in recognizing them at recovery. Dissection for implant recovery included identification of all regions of host fat body as well as donor tissue to assure that host adipose tissues had not been injured during implantation.

Figure 1 summarizes the outline of the experiments and data from studies with normal (intact, undamaged), mechanically-injured, and collagenase-treated implants. Intact *Ore-R* region 6 fat bodies remained normal and unencapsulated in *ts* hosts. The cells of these implants appeared healthy and the tissue surfaces, like the hosts' fat bodies, were free of adhering blood cells (Fig. 2). TEM examination of groups of donor tissues at the time of implantation and after recovery from larval hosts confirmed the integrity of the basement membrane. Fat bodies injured prior



Figs. 2 and 3. Sample dissections of *ts* host fat body and recovered implants. All regions of host fat body are accounted for including region 6s (*h*). The region 6 implant (*i*) in Fig. 2 was intact at the time of implantation, remained intact and has no lamellocytes on its surfaces. Injured implanted fat body (*i*) in Fig. 3 has been encapsulated and melanized. Feulgen whole mount preparations; salivary gland (*s*). Scale – 1 mm

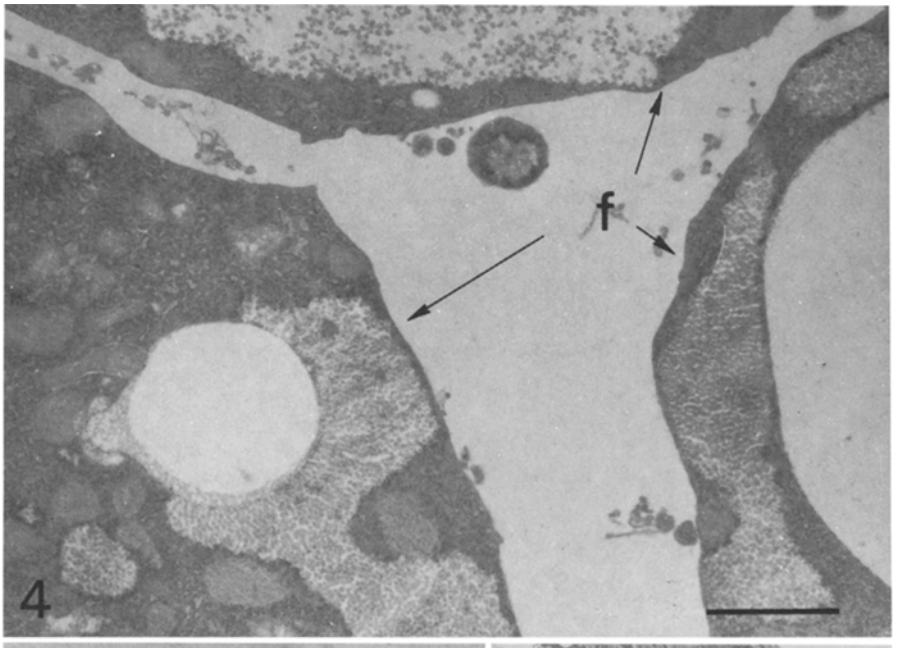
to implantation were invariably encapsulated and melanized (Fig. 3). Hosts receiving these implants generally had a number of melanotic masses rather than a single, large mass. The melanotic masses were often visible by external examination of hosts, but dissections were completed in all cases to be certain that the melanized masses were implanted fat bodies and not damaged host tissues. Examination of the melanized implants verified that they were masses of adipose cells (alveolar cytoplasm and nuclei with polytene chromosomes) encapsulated by layers of lamellocytes. It should be noted that in five cases intact implants had a small melanized spot and adhering blood cells at the neck regions. Considering the results with injured tissues, these cases probably represent localized injury inflicted during separation of the region 6s for implantation.

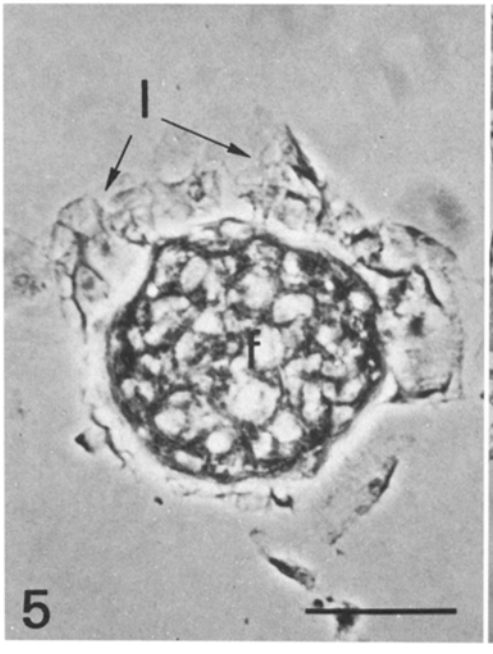
Hosts receiving collagenase-treated implants had numerous, minute black specks scattered throughout the body. To verify that these small bodies were implanted adipose cells, some of them were collected and processed for TEM. Examination of these implants showed adipose cells encapsulated by lamellocytes; collagenase-treated region 6 cells prior to implantation were also examined by TEM to confirm that basement membrane had been removed (Figs. 4–6).

Heterospecific Fat Body Implants in ts Hosts

Since the *ts* hemocytes encapsulate damaged fat body surfaces and fat body cells without basement membrane but not intact fat body surfaces, it is clear that the encapsulation response is elicited against tissues whose surfaces are not covered by normal basement membrane. The next question was whether the hemocytes can recognize normal, undamged tissue surfaces from other *Drosophila* species as "not self". The same protocol as above was used to implant intact region 6 fat bodies from five sibling species of *D. melanogaster* and fat bodies from five more distantly related species into *ts* hosts. In these experiments hosts were not dissected for implant recovery until shortly after pupariation unless melanized implants were visible earlier.

Fat bodies from the five sibling species of the *D. melanogaster* species subgroup remained healthy and unencapsulated in *ts* hosts whereas fat bodies from the other species were encapsulated (Table 1, Sect. A; we have no explanation for the single exception in *D. nebulosa* other than the possibility of excessive loss of lamellocytes along with hemolymph during implantation). Encapsulation of *D. virilis* and *D. willistoni* tissues was generally more rapid than encapsulation of tissues from the other species and melani-





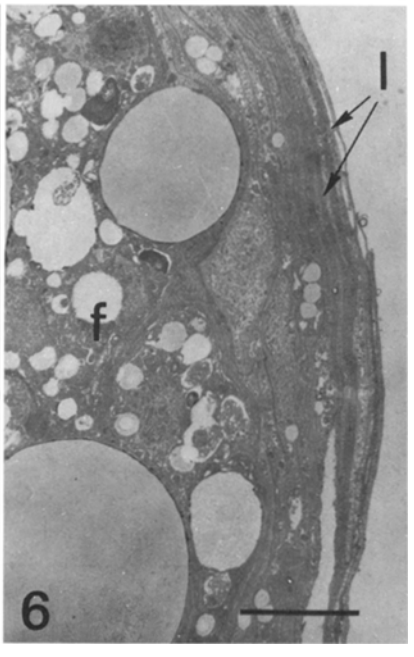


Fig. 4. Electron micrograph of an epon section through the surfaces of three region 6 fat body cells (*f*) following collagenase treatment to show that basement membrane has been removed; this control represents the condition of the cells at the time of implantation. Scale – 1 μm

Fig. 5. An implanted adipose cell (*f*) from collagenase-treated region 6 *Ore-R* fat body recovered from a *ts* host larva. This cell is surrounded by lamellocytes (*l*) adhering to its surfaces. Feulgen; phase contrast. Scale – 50 μm

Fig. 6. Electron micrograph showing the surface of an encapsulated adipose cell (*f*) from a collagenase-treated region 6 fat body and the layered wall of lamellocytes (l). Scale – 8 μm

zation of these implants was often apparent before pupariation. Tissues from *D. nebulosa* and *D. pseudoobscura* often remained unmelanized until pupariation. Unlike the mechanically-injured tissues, heterospecific implants were recovered as complete fat bodies encapsulated by lamellocytes. The possibility that heterospecific tissues were adversely affected by the experimental manipulations was ruled out by implanting *D. virilis* region 6 fat bodies into *D. virilis* hosts. These implants remained intact and healthy whereas groups of *D. virilis* fat bodies implanted simultaneously into *ts* hosts were encapsulated and melanized.

The condition of *D. virilis* implants in *ts* hosts was also examined by electron microscopy. For these studies groups of implants were recovered at intervals between 0 and 48 h after implantation. By examining at least five implants at various times after implantation it was determined that the encapsulation response of the *ts* host lamellocytes commences about 14 h after implantation, by 24 h the implants are fully encapsulated, and by 48 h melanization is underway

Table 1. Heterospecific implants in *ts* larval hosts

	Encapsulated	Unencapsulated
A. Fat bodies		
Sibling species:		
D. erecta	0	24
D. mauritiana	0	15
D. simulans (c)	0	15
(c135.20)	0	8
D. teissieri	0	17
D. yakuba	0	17
Other species:		
D. willistoni	22	0
D. nebulosa	15	1
D. novamexicana	24	0
D. pseudoobscura	19	0
D. virilis	92	0
B. Imaginal disks		
D. virilis	27	0
D. simulans	0	19

(Figs. 7 and 8). Examination of the ultrastructure of the donor tissues showed that the basement membranes were intact (Fig. 9). Unlike the collagenase-treated cells and the injured *Ore-R* tissues, there were no intercellular gaps between the encapsulated cells showing infiltration of lamellocytes nor did the donor fat body cells show any rounding up as in the case of the affected cells in *tu-W* larvae (Rizki and Rizki 1979). The intracellular contents did not reflect any unusual features and the ER, mitochondria, glycogen, lipid droplets, and intranuclear structures were discernible in cells of tissues recovered prior to encapsulation as well as in encapsulated cells of the 24 h specimens.

Imaginal Disk Implants in ts Hosts

The fat body is a larval tissue with nondividing cells and polytene chromosomes. Its surfaces are covered by basement membrane similar to that of the imaginal disks which consist of mitotically active cells. To determine whether surfaces of these two types of tissue differ with regard to the encapsulation reaction, we implanted wing and leg imaginal disks from *D. virilis* and *D. simulans* larvae in *ts* hosts of the same ages as in the studies with the fat body tissues. The results paralleled those obtained with larval fat bodies: *D. virilis* imaginal disks were encapsulated and melanized in *ts* hosts whereas *D. simulans* imaginal disks were not *(Table 1, Sect. B)*.

Ore-R Larval Hosts

A final series of implantation studies was undertaken to evaluate the fate of *D. virilis* tissues in *Ore-R* (ts^+) larvae that do not show the precocious presence of lamellocytes. Ages of donors and hosts, experimental procedures and temperature were comparable to those used in the studies with *ts* larvae.

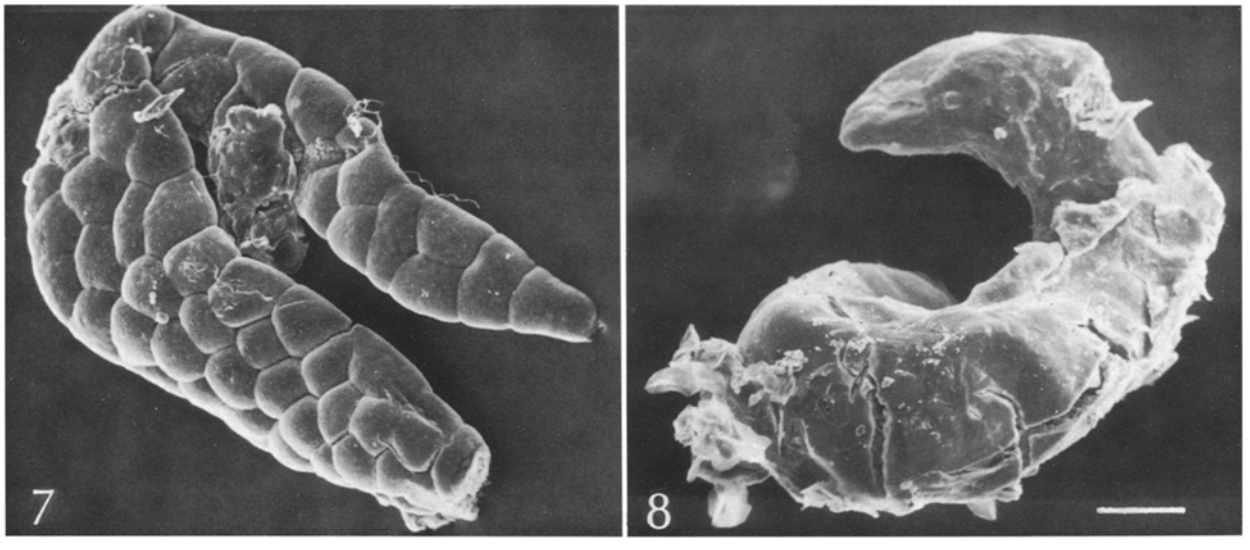


Fig. 7. A region 6 of *D. virilis* fat body recovered from a *ts* host after 8 h at 18° C. There are no blood cells on the tissue surfaces. This piece of tissue and that in Fig. 8 were individually handled for critical point drying and mounting on aluminum stubs for SEM; the handling is responsible for the cracks in the specimens

Fig. 8. A fully encapsulated and melanized region 6 *D. virilis* fat body recovered from a *ts* host after 48 h at 18° C. The tissue is completely enclosed by the lamellocyte layers. Scale – 50 μm

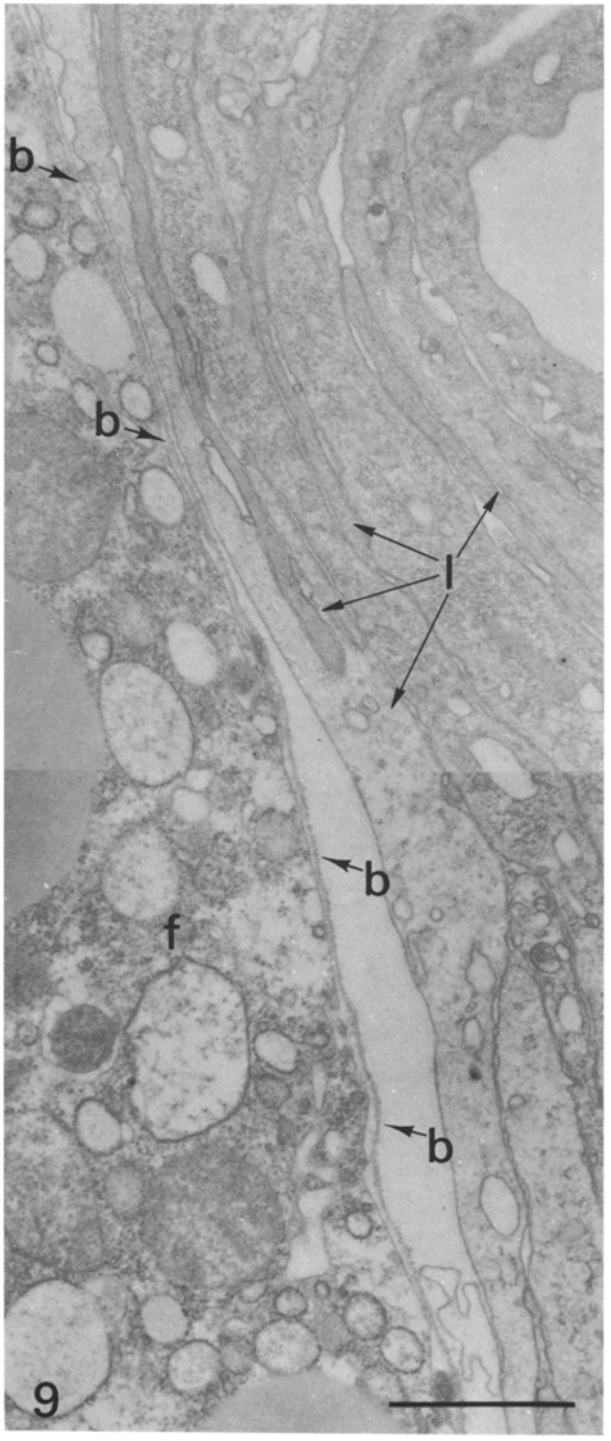


Fig. 9. Section passing through the zone of encapsulating lamellocytes (*l*) on a *D. virilis* region 6 implanted fat body (*f*). This region with the slight gap between the two cell types was chosen to illustrate the integrity and continuity of the basement membrane (*b*) underneath the lamellocyte layers. Scale – 1 μm

Region 6 fat bodies of *D. virilis* (23 implants) remained intact and unmelanized in *Ore-R* hosts through pupariation. Since the cells of the larval fat body are dispersed as single cells during the early pupal period, this tissue is unsuitable for assessing the fate of implants after this time in *Ore-R* hosts. Imaginal disks implanted into *Drosophila* larvae will survive metamorphosis and are recoverable in the hatching adults (Beadle and Ephrussi 1935). Therefore, we implanted *D. virilis* wing and leg imaginal disks into *Ore-R* hosts, and as controls for these series had implanted *D. virilis* imaginal disks in *ts* hosts.

The *D. virilis* imaginal disks (7 implants) underwent metamorphosis in the *Ore-R* hosts and showed cuticular structures of the notum with wing blade and legs when recovered in adult flies. The *D. virilis* imaginal disks (7 implants) recovered from *ts* adults were covered with lamellocytes, melanized and showed no sign of differentiation. Imaginal disk tissues were not processed for electron microscopy so additional details regarding tissue surfaces are not yet available.

Discussion

The implantation studies in this report demonstrate that *ts* lamellocytes at 18° C are capable of engaging in encapsulation to form melanotic capsules. That host posterior fat bodies remained unencapsulated in these experiments indicates they were not appropriate sites for encapsulation activity, and confirms that the absence of melanotic tumors in *ts* larvae at 18° C is a result of normalization of fat body surfaces (Rizki and Rizki, preceding paper). Furthermore, *Ore-R* fat body implants with intact basement membrane remained free of lamellocytes in *ts* hosts while *Ore-R* tissues with damaged surfaces or without basement membrane were reacted against by *ts* lamellocytes. These results agree with Salt's (1970) reasoning in summarizing the transplantation studies with a variety of insect hosts. Salt concluded that insect blood cells will remain neutral to implanted tissues covered by homologous membranes whereas foreign objects excite an encapsulation reaction since they lack this appropriate covering. Scott (1971) extended this concept by demonstrating that cockroach homologous nerve cords were susceptible to encapsulation after digestion with collagenase and lecithinase C. In the present study TEM examination of the collagenase-treated cells prior to implantation verified the absence of basement membrane and the integrity of the adipose cell membranes.

The encapsulation of intact heterospecific tissues by *ts* lamellocytes implies the surfaces of these implants were recognized as "not self". TEM examination showed the presence of basement membrane on the *D. virilis* implants so distinction of "not self" from "self" must reside in the molecular architecture of the basement membrane. It is difficult to exclude

the possibility of metabolite diffusion from within the implanted tissues as attractants for hemocyte activity, but such an explantation seems unlikely when considering that the implanted tissues are constantly bathing in circulating hemolymph. Since the *ts* lamellocytes did not encapsulate tissues of sibling species, either their discriminatory power is limited or the basement membranes of the sibling species have not undergone critical changes in the course of their molecular evolution. The present survey did not include tissues from the other subgroups of the *melanogaster* group and it will be interesting to test tissues from some of these species in *ts* hosts. Another possibility to be examined is the existence of mutants affecting transplant histocompatibility in *D. melanogaster*.

The availability of lamellocytes, or the capability of the plasmatocytes to assume the lamellocytic form, is a crucial factor in the encapsulation response since the cellular walls of the capsules consist of these flattened cells. Both larval tissue and imaginal disks of *D. virilis* were consistently encapsulated in *ts* hosts demonstrating they are adequate for stimulating an encapsulation reaction when lamellocytes are present. These same tissues remained unencapsulated in *Ore-R* larval hosts lacking precociously differentiated lamellocytes so lamellocytes must be present in the host for successful encapsulation. This explains Bodenstein's (1956) finding that *D. virilis* imaginal disk tissues thrive in *D. melanogaster* hosts. However, *Ore-R* larvae which do not have lamellocytes in circulation most of their larval life can develop lamellocytes following experimental manipulations (Rizki 1962). Whether these cells will respond like *ts* lamellocytes remains to be investigated as do the factors that control the transition in morphology of the blood cells.

Additional factors to be considered in the cell-mediated defense response include the nature of the foreign surfaces and the response time. Phagocytic reaction to *Escherichia coli* injected in the larval hemocoel requires a few minutes (Rizki and Rizki 1980; unpublished observations). These injected bacteria adhere not only to the surfaces of phagocytic forms (plasmatocytes and podocytes) but also to the surfaces of lamellocytes in circulation or stationary on the surfaces of melanotic tumors. Thus, lamellocytes show prompt adhesivity for some foreign materials, but their adhesive behavior against *D. virilis* tissues is delayed for hours.

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