Haemocyte changes in resistant and susceptible strains of *D. melanogaster* caused by virulent and avirulent strains of the parasitic wasp *Leptopilina boulardi*

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Abstract

Two strains of *Drosophila melanogaster* (resistant and susceptible) were parasitized by a virulent or avirulent strain of the parasitoid wasp *Leptopilina boulardi*. The success of encapsulation depends on both the genetic status of the host strain and the genetic status of the parasitoid strain: the immune cellular reaction (capsule) is observed only with the resistant strain–avirulent strain combination. The total numbers of host haemocytes increased in all 4 combinations, suggesting that an immune reaction was triggered in all hosts. Resistant host larvae infected with the virulent or avirulent strains of parasitoid wasp had slightly more haemocytes per mm$^3$ than did susceptible host larvae at the beginning of the reaction (less than 15 h post-parasitization). This difference disappeared later. Only the virulent parasitoid strain caused the production of a high percentage of altered lamellocytes (from a discoid shape to a bipolar shape), half the total number of lamellocytes are altered. This suggests that the alteration of lamellocyte shape alone is not sufficient to explain the lack of capsule formation seen in resistant hosts parasitized by the virulent strain. Lastly, there were very few altered lamellocytes in resistant or susceptible hosts parasitized by the avirulent parasitoid strain, two combinations in which no capsule was formed. As is now established for *Drosophila*-parasitoid interactions, virus-like particles contained in the long gland of the female wasp affect the morphology of the lamellocytes. The results presented here are further proof of the action (direct or indirect) of virus like particles of the virulent strain on lamellocytes. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: *Drosophila*; Parasitoid; Resistance; Virulence; Immune response; Haemocyte

1. Introduction

The main haemocyte-mediated defence of insect larvae against parasitoids is encapsulation (Ratcliffe, 1993; Carton and Nappi, 1997). This cellular immune reaction is influenced by the genetic and physiological parameters of the host and the parasitoid. The number and type of the host haemocytes are two of the key factors required for a successful immune reaction (Eslin and Prévost, 1998). The parasitoid may have protecting virus-like particles and polydnaviruses (for a review, see Vinson, 1993; Beckage, 1998). These are introduced into the host hemocoel by the adult female wasp and block the host immune reaction by profoundly affecting the integrity of the host haemocytes (Rizki and Rizki, 1984; Strand and Pech, 1995).

*Leptopilina boulardi* is a parasitoid wasp (Figitidae, Hymenoptera) that parasitizes the larvae of *Drosophila melanogaster* (Carton et al., 1986). The *Drosophila* larvae can develop a cellular capsule that kills the parasitoid (Carton and Nappi 1991, 1997), but certain strains of *Leptopilina* can overcome this reaction (Dupas et al., 1998; Dupas and Carton, 1999; Vass et al., 1993) and prevent encapsulation (these strains are called virulent strains).

It has long been recognized that capsule formation around parasitoid eggs in *Drosophila* starts with an
increase in the number of circulating haemocytes (Rizki, 1957; Walker, 1959; Nappi and Streams, 1969). This is the first event in a cellular immune reaction (Carton and Kitano, 1979; Brehelin, 1982; Nappi and Carton, 1986; Rizki and Rizki, 1992; Eslin and Prevost, 1996). The capsule is formed by the deposition of several layers of transformed haemocytes, the lamellocytes. Virus-like particles are responsible for the virulence of Leptopilina heterotoma (Rizki and Rizki, 1990) and Leptopilina bouardi (Dupas et al., 1996) towards their Drosophila hosts. Rizki and Rizki (1994) found that these particles adhere to the lamellocytes. Parasitization by L. heterotoma or L. bouardi (Rizki et al., 1990) alters the lamellocytes so that the normal disc-shaped cells become bipolar.

We have now compared the changes that occur in the hemograms of resistant and susceptible hosts infested by avirulent or virulent strains of L. bouardi. Only one combination (resistant host strain–avirulent strain of parasitoid) results in all the eggs being encapsulated. The three other combinations result in no egg being encapsulated (Russo et al., 1996; Dupas et al., 1996), whereas the total number of haemocyte in the hemolymph increased in all four combinations of infestation. The success of encapsulation depends on both the genetic status of the host strain and the genetic status of the parasitoid strain.

2. Materials and methods

2.1. Animals and parasitization of host larvae

The strains of D. melanogaster used were the resistant (R) strain 1088, selected for its resistance to L. bouardi, and the susceptible (S) strain 1089 that has no immunity to L. bouardi. Second instar larvae were parasitized by the virulent (V) strain G464 or by the non-virulent (Av) strain G 486 of L. bouardi. The eggs of the V strain are never encapsulated, whereas those of the Av strain are encapsulated by the R strain hosts. D. melanogaster were grown on standard cornmeal and yeast medium at 25°C. Adult wasps were fed a 50% honey solution and kept at 18°C. Larvae were infected by exposing 10 host larvae (48 h old, second instar larvae) to 5 female wasps for 2 h. Experiments were done twice (each batch corresponded to 10 larvae submitted to parasitization) for each combination (Table 1). Reference strain numbers are from the laboratory stock at Gif-sur-Yvette (France).

2.2. Hemograms

The total number of haemocytes per mm$^3$ (THC) and the numbers of each type of haemocyte per mm$^3$ were determined at 6, 15 (second instar), 24 and 48 h (third instar) after parasitization (Russo et al., 1996). Observations made over 48 h after parasitization could be mis-interpreted, since they correspond to prepupal or pupal stages, with tissue lysis.

Hemolymph was collected directly from each larva in a Thomas hemocytometer (0.02 mm depth), without dilution, and haemocytes were counted under a phase contrast microscope. Each larva was then dissected to determine the number of eggs deposited by the wasp. The detailed results are given only for single parasitism (one egg per larva). The haemocyte types were defined according to Brehelin (1982). Only lamellocytes and plasmatocytes were examined because the third type of haemocyte, crystal cells, account for only 3–5% of the THC (Brehelin, 1982) and the number of crystal cells does not change following parasitization (Carton and Kitano, 1979). The modified lamellocytes (Lm) with a bipolar shape and blunt or flared ends (instead of the normal disc shape) occurring after parasitization were observed and compared using ANOVA.

3. Results

3.1. Genetic aspects of the four host-parasitoid combinations studied

The occurrences of egg encapsulation in the 4 combinations used here (Russo et al., 1996) are summarized in Table 1. The resistance of D. melanogaster to avirulent parasitoid strains is autosomal and monogenic (Carton et al., 1992; Benassi et al., 1998). One major gene ($\text{Rib}^+$) confers resistance to L. bouardi (Hita et al., 1999; Poirier et al., 2000). A gene that controls the immune suppression ($\text{Ism}^+$) of D. melanogaster has also been identified in virulent strains of L. bouardi (Dupas et al., 1998). Resistant and susceptible strains have the following

<table>
<thead>
<tr>
<th>L. bouardi</th>
<th>Avirulent strain</th>
<th>Encapsulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rlb+/Rlb+</td>
<td>Ism+/Ism+</td>
<td>No capsule</td>
</tr>
<tr>
<td>Rlb+/Rlb-</td>
<td>Ism-/Ism-</td>
<td>No capsule</td>
</tr>
</tbody>
</table>

Table 1 The four combinations of parasitization: immune responses (capsule formation) of resistant and susceptible host strains (D. melanogaster) parasitized by virulent or avirulent strains of parasitoid (L. bouardi). The resistance of D. melanogaster to avirulent L. bouardi strain is autosomal and monogenic. One major gene ($\text{Rib}^+$) confers resistance to L. bouardi. A gene that controls the immune suppression ($\text{Ism}^+$) of D. melanogaster has also been identified in virulent strains of L. bouardi.
L. boulardi per mm$^3$ (THC) and numbers of lamellocytes in the con-

Dupas and Carton, 1999). The total haemocyte counts

Ism

3.2. Changes in total haemocyte counts

The THC 6 h after parasitization was significantly higher than in controls in all 4 combinations of parasitization (Table 3). The maximum THC was reached earlier (15 h after parasitization) with the R strain rather than with the S strain (24 h after parasitization). There was no difference between the V and Av parasitoid strains, except for the S–V strain interaction, the maximum THC reaching 24 h after parasitization instead of at 15 h, as with the parasitoid Av strain. The number of lamellocytes was decreased at 48 h after parasitization in all the host-parasitoid strain combinations, but remained significantly higher than those of controls (Table 2).

THC values in superparasitism were significantly elevated (results not shown) for each of the 4 combinations as early as 6 h after infestation. Maximum THC reached 25257 per mm$^3$ and appeared to be 1.5 times higher than in single parasitism after the same interval (15 h).

3.3. Changes in the number of lamellocytes per mm$^3$

There were few lamellocytes in control host larvae at the start of the experiments (2nd instar larvae) and there was no difference between the R and S strains of hosts (Table 2). The relative percentage of lamellocytes (compared to THC) and Lm (compared to lamellocyte count) in infested larvae are given in Table 4. The number of lamellocyte per mm$^3$ in infested larvae was always significantly higher than in control larvae of the same age. All the combinations had significantly increased numbers of lamellocytes at 15 h post-parasitization, with the maximum reached at 24 h. This number of lamellocytes could be 15–20 times that in the hemogram of 6 h larvae. The difference was significant only at 15 h with R strain infested by V strain, compared to parasitization by Av strain, but this difference was not maintained after 15 h. The number of lamellocytes was decreased at 48 h in all combinations. The relative frequency of lamellocytes in R and S strains infected with the Av strain was higher than in the same hosts infected with V strain at 15 h (22.8% >8.2% and 42.6% >21.4%, respectively) and at 24 h (51.7% >30.9% and 35.2% >25.0%, respectively) (Table 4).

Superparasitism (results not shown), resulted in a significant increase in the number of lamellocytes in both R and S strains superparasitized by Av or V parasitoid strains as early as 6 h. However, this increase was less rapid with the V strain of parasitoid; the maximum was reached 24 h after parasitization instead of at 15 h, as with the parasitoid Av strain. The number of lamellocytes also decreased more slowly than in single parasitism.

3.4. Changes in lamellocytes morphology

We found no lamellocyte with modified morphology (Lm) in the controls. Lm appeared at 6 h with R and S strains, but only when the L. boulardi strain was virulent (Table 4). The maximum percentage of Lm (35–60%), calculated from the total lamellocyte number, was reached at 15 h and remained throughout the development of the Drosophila infested larvae. In contrast, Lm remained very rare in hosts infected with the Av strain (<3%).
Table 4
Total numbers of lamellocytes and modified shape lamellocytes (Lm) per mm$^3$ in single parasitized larvae, 6 h, 15 h, 24 h and 48 h after parasitization. Results are means of counts from 10 larvae followed by the standard deviation. Values with the same index (Greek letter) in the same table are not significantly different (at 95% level). Lamellocyte counts are also given as the % of THC and Lm are also given as the % of the lamellocyte count$^a$

<table>
<thead>
<tr>
<th>D. melanogaster</th>
<th>Resistant strain</th>
<th>L. boulardi</th>
<th>Susceptible strain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lamellocyte</td>
<td>Lm</td>
<td>Lamellocyte</td>
</tr>
<tr>
<td>6 h after infestation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L.b. Av strain</td>
<td>252±24$^a$ (2.8%)</td>
<td>0 (0%)</td>
<td>152±93$^a$ (2.1%)</td>
</tr>
<tr>
<td></td>
<td>229±24$^a$ (3.1%)</td>
<td>24±10$^a$ (10.5%)</td>
<td>210±17$^a$ (3.3%)</td>
</tr>
<tr>
<td>15 h after infestation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L.b. Av strain</td>
<td>406±752$^a$ (22.8%)</td>
<td>0 (0%)</td>
<td>3572±703$^a$ (42.6%)</td>
</tr>
<tr>
<td></td>
<td>1512±496$^a$ (8.1%)</td>
<td>887±264$^a$ (58.7%)</td>
<td>1813±261$^a$ (21.4%)</td>
</tr>
<tr>
<td>24 h after infestation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L.b. Av strain</td>
<td>4921±731$^a$ (51.7%)</td>
<td>32 ± 22$^a$ (0.6%)</td>
<td>5499±903$^a$ (35.2%)</td>
</tr>
<tr>
<td></td>
<td>3240±753$^a$ (30.9%)</td>
<td>546±162$^a$ (16.8%)</td>
<td>2322±463$^a$ (25.0%)</td>
</tr>
<tr>
<td>48 h after infestation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L.b. Av strain</td>
<td>1809±339$^a$ (19.3%)</td>
<td>35±20 (1.6%)</td>
<td>183±433$^a$ (27.2%)</td>
</tr>
<tr>
<td></td>
<td>2096±284$^a$ (24.3%)</td>
<td>857±264 (40.9%)</td>
<td>2091±375$^a$ (24.2%)</td>
</tr>
</tbody>
</table>

$^a$ L.b.: L. boulardi; Av: avirulent; Lm: modified shape lamellocyte; R: resistant; S: susceptible; V: virulent.

4. Discussion

The data on the effect of parasitism on the total and differential haemocyte counts show great variations, depending on the insect species studied (see Stettler et al., 1998 for a review). The increase in the THC that occurs in infested Drosophila larvae (Rizki and Rizki, 1984, 1992; Eslin and Prévost, 1998) is one of the first indications of an immune reaction but a temporary decrease following parasitization has been also observed in some other insect species (Strand and Pech, 1995; Bauer et al., 1998).

The THC and the number of lamellocytes in the R and S larvae of D. melanogaster before parasitization are similar. Hence, the striking difference in the cellular immune responses (capsule formation) of these two host strains infested by the Av strain of L. boulardi is not due to a difference in the THC of the host larva before parasitization (Carton and Nappi, 1997). We found increases in THC and lamellocytes after parasitization with all the combinations tested. The THC for the R-Av combination, in which there was capsule formation, was the same as in the R-V combination, in which there was no capsule formation. The changes occurring to the haemocytes in the S strain after parasitization by either Av or V strains of L. boulardi were similar, but less marked. The numbers of lamellocytes and Lm were similar in the R-Av combination (capsule formation) to those in the S-Av combination (no capsule formation) for single parasitism. The changes in superparasitized hosts were very similar. Consequently, the maximum THC does not depend on further capsule formation.

This raises the question of what factor triggers the increase in THC. Previous studies have shown that there is a very small increase in the THC or lamellocyte counts in D. melanogaster larvae after a discrete puncture of the cuticle or the injection of buffer. But there is a response to the injection of particulate material (Brehelein, 1982) or parasitization (Rizki and Rizki, 1992). The latter authors conclude that the injury inflicted by the female ovipositor is not solely responsible for the increased THC in parasitized larvae. The formation of a capsule (or lack of capsule) by R and S strains of D. melanogaster parasitized by the V or Av strains of L. boulardi cannot be deduced from the change in the hemogram profile that occurs for the four combinations.

Hence, the main reason for the increase in the THC and lamellocytes is probably the presence of an egg and/or of the material (virus-like particles, venom) injected with the egg. The host recognizes an aggression and launches the main constituent of the cellular defense reaction, an increase in the total haemocyte account. It has been shown (Carton and Kitano, 1979) that the number of crystal cells does not vary with infestation. Recently, Braun et al. (1998) demonstrated that melanization could occur in domino mutant Drosophila larvae that are devoid of blood cells.

As Rizki and Rizki (1994) stated, the lamellocytes of parasitized D. melanogaster larvae lose their discoid shape and become bipolar. The change in cell morphology is correlated with changes in cell adhesion. Normal lamellocytes are sticky cells that adhere and clump together, whereas Lm remains solitary (Rizki et al., 1990). While the numbers of Lm in R and S strains infected with Av strains are low and similar, the results are sharply different in hosts infected with the V strain of L. boulardi. It increases from about 0–64 Lm per mm$^3$.
to 612–861 Lm per mm³. More than 35–50% of the lamellocytes are converted to bipolar lamellocytes following infection with a V strain. The morphology of the lamellocytes is altered by a virus-like factor injected along with the egg by the parasitoid female (Rizki and Rizki, 1990). The virus-like factor is present in the female reproductive tract of L. boulardi and is injected with the egg into Drosophila larvae (Dupas et al., 1996; Russo et al., 1996). Hence, our results are further proof of the action (direct or indirect) of virus-like particles on lamellocytes. An altered lamellocyte morphology is the most evident effect of infection by a V strain of the parasitoid (Rizki and Rizki, 1990). But even in a V strain infestation, which produces many more Lm than does an Av strain infestation, fewer than a half of the total lamellocytes are altered. This morphological alteration of lamellocytes is not sufficient to explain the lack of capsule formation; other changes in lamellocytes have been observed (Rizki and Rizki, 1994). These could be changes in the cell surface properties, as suggested by Nappi and Silvers (1984). This agrees with our conclusion that a lack of encapsulation is due to active protection triggered by the L. boulardi V strain, rather than to passive prevention of an immune reaction. This is confirmed by the fact that R strain hosts parasitized by both V and Av strains simultaneously do not encapsulate the Av egg which appears to be cross protected by co-injected factors with the egg of the V strain from the host defence reaction (Carton, unpublished results). This intra-specific level findings confirms the results of Rizki and Rizki (1984) and Nappi and Silvers (1984) on the cross protection between two parasitoid species with different status of virulence parasitizing the same host larvæ.

Two main conclusions can be drawn from these results. First, that THC increased in all the combinations, whatever the genetic status of the host or parasitoid strain; second, only the virulent parasitoid strain causes the production of a high percentage of altered lamellocytes. Cellular resistance appears to have two components (Fellowes et al., 1999): a general increase in circulating haemocytes and a specific recognition of the egg associated with a melanization. Encapsulation takes place independently of any increase in the number of hemocytes for each genetic combination. As suggested with the two-components model, the increase in hemocyte number is necessary (prerequisite) for an efficient immune response. Other factors are needed, especially to explain the specificity of this cellular reaction, which occurs only with the R-Av combination. This specific component could involve a specific recognition process that develops only in the R-Av combination; the specific gene of resistance Rib may play a major role in this process (Carton and Nappi, 1997; Poirié et al., 2000). Altered lamellocytes are found after parasitization with the virulent strain, even if this alteration is not the only source of protection. Here again, the genetic status of the parasitoid strain plays a major role in the alteration of the hemocytes.

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