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Developmental variability of the antibacterial response in larvae and pupae of *Calliphora vicina* (Diptera: Calliphoridae) and *Drosophila melanogaster* (Diptera: Drosophilidae)

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Insect immunity, antibacterial peptides, pupal development, ecdysteroids, *Calliphora*, *Drosophila*

Abstract. The inducibility of antibacterial peptides in *Calliphora* is strongly reduced during pupal development. Similarly, a downregulation of the inducibility of a diptericin reporter transgene is observed in the pupae of transgenic *Drosophila*, indicating existence of a negative correlation between inducibility and ecdysteroid titre. In the present paper we show that the level of antibacterial activity induced by the injection of bacteria into the diapausing larvae of *Calliphora* is reduced if the insects simultaneously receive 20-hydroxyecdysone. The same effect is observed in ligated *Calliphora* larvae deprived of their brain-retrocerebral complex. The data suggest that at certain developmental stages ecdysteroids downregulate the induced antibacterial defense.

INTRODUCTION

The humoral response of insects to an immune stress consists of rapid and transient synthesis of a large variety of antibacterial peptides (for review, see Hoffmann et al., 1993; Hultmark, 1993; Cociancich et al., 1994). These antibacterial peptides are mainly synthesized in the fat body and are subsequently secreted into the hemolymph where they act on Gram-negative and/or Gram-positive bacteria. Most biochemical purifications of these peptides have been performed using bacteria-challenged larvae or adults where a strong antibacterial response can be triggered; diapausing pupae have been used only in a few cases, as in the case of the discovery of cecropins in the lepidopteran *Hyalophora cecropia* (Hultmark et al., 1980; Steiner et al., 1981).

Fragmentary data point to a reduced antibacterial response in immune-challenged pupae. This has been reported in *Drosophila* with the analysis of cecropin and diptericin transcripts (Samakovlis et al., 1990; Wicker et al., 1990). We set as our goal to identify the mechanism of reduced antibacterial response in the pupal development of two dipteran insects: *Calliphora vicina* and *Drosophila melanogaster.*

MATERIALS AND METHODS

Insects

Experiments were performed with a wild type laboratory strain of *Calliphora vicina* collected from the Murmansk region, Northern Russia (gift of Dr Vinogradova, Zoological Institute, St-Petersburg). The peculiarity of this strain is its relatively stable larval diapause (Chernysh & Simonenko, 1988; Vinogradova, 1991). To induce diapause parent, adult flies were kept under short day conditions (12L : 12D); larvae

were grown in short day at 12°C and placed to 3°C at the end of the feeding period; at this temperature they were kept for 2 months until the beginning of our experiments. All experiments were performed at 20°C. Under these conditions, the pupal stage lasted about two weeks.

When appropriate, larvae were ligatured at one third of their body length to isolate the brain and ring gland from the rest of the body.

The transformed *Drosophila* fly line Dipt2.2-*lacZ*:1 (Reichhart et al., 1992) was maintained at 25°C on a standard cornmeal medium. In these conditions, pupariation occurs at 120 h after egg-laying, and adults emerge at 240 h.

Bacterial challenge

Calliphora: To induce an immune response, larvae and pupae were pricked with a needle previously dipped into a suspension of heat-killed *Escherichia coli* D31 (ca. 10^9 bacteria/ml) and left overnight. Their surface was then sterilized in 96% ethanol, washed with distilled water, dried and hemolymph of 10 individuals (approximately 10 µl per animal) was collected in ice-cold tubes containing a protease inhibitor (aprotinin; Sigma) at a final concentration of 20 µg/ml. Three identical batches were prepared for each experimental group. Hemolymph was centrifuged at 12,000 rpm for 30 min at 4°C. The supernatant, referred to as plasma, was frozen at –70°C until further use.

For evaluation of the effects of 20-hydroxyecdysone (20E, Sigma), 16 diapausing larvae in each experimental group were injected with 1 µl of a solution containing 3.6×10^5 heat-killed *E. coli* D31 and various concentrations of 20E, and left at 20°C. The next day, two 2-µl aliquots of hemolymph were taken from each larva and tested for antibacterial activity.

Drosophila: Larvae and pupae were pricked with a tungsten needle dipped into a bacterial suspension (*E. coli* 1106 and *Micrococcus luteus* A270), and maintained at 25°C for various time intervals.

All bacterial strains were from the Laboratory of Insect Immune Response and Development, Institute of Molecular and Cell Biology, Strasbourg.

Antibacterial assays

Solid growth inhibition assay conditions were essentially those described by Lambert et al. (1989) with the difference that the plasma sample was directly placed on the surface of the agar plate instead of being deposited into wells. Liquid growth inhibition assay conditions were those described by Bulet et al. (1993).

β-Galactosidase assay

Five individuals of *Drosophila* larvae or pupae were homogenized in 60 mM $Na_2HPO_4.7H_2O$, 40 mM $NaH_2PO_4.H_2O$, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol (pH 7.0). β-Galactosidase activity was measured using O-nitro-phenol-β-D-galactoside as substrate (Herbomel et al., 1984) and was expressed as nmol of product that appeared per min. per mg of protein.

RESULTS

Variability of the antibacterial response during pupal development of *Calliphora*

Before testing the appearance of antibacterial activity in *Calliphora* pupae in response to an immune challenge, we looked for the possible presence of constitutive antibacterial activity. We were unable to detect any activity, either against *E. coli*, or against *M. luteus*, in plasma samples from 1- to 4-day-old pupae (data not shown).

In contrast, plasma samples from 24-h bacteria-challenged pupae of various ages contained both Gram-negative (*E. coli*) and Gram-positive (*M. luteus*) activity. The results, illustrated in Figure 1, show that the capacity to produce antibacterial molecules following an immune challenge drastically depends on the developmental stage. The highest activities were found in stimulated early pupae (day 0 and day 1). Activities significantly decreased during later stages. The production of anti-Gram-positive molecules in response to challenge dropped to low levels by mid-pupal development (days 6–8), whereas anti-Gram-negative material was almost undetectable from day 2 onwards. Anti-Gram-

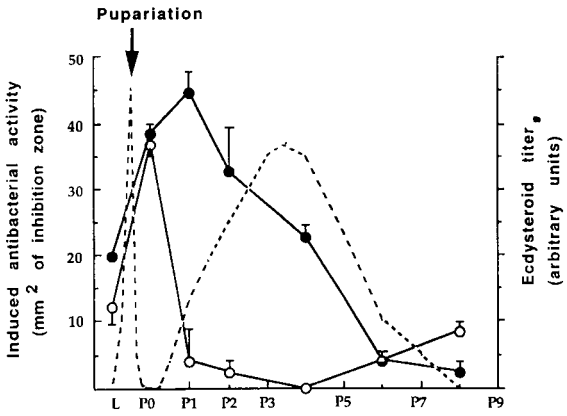

Fig. 1. Profile of antibacterial activity in the hemolymph of bacteria-challenged larvae and pupae of *Calliphora vicina*. The anti-Gram-negative (○) and anti-Gram-positive (●) activities are given as inhibition zone areas in a solid growth inhibition assay (in mm^2). The dotted curve represents the ecdysteroid titer in the hemolymph of *Calliphora vicina*, redrawn after Shaaya & Karlson (1965) in arbitrary units. The data are presented as mean ± SE. L – larvae; P – pupae.

positive activity slightly went up after day 6. Recovery of antibacterial activity at the end of pupal stage when the hemolymph ecdysteroid titer is low was confirmed in additional experiments (data not shown).

Figure 1 also presents the hemolymph titer ecdysteroids according to Shaaya & Karlson (1965). There is clearly a negative correlation between ecdysteroid titers and inducible antibacterial activities.

Variability of the inducibility of a diptericin reporter gene during pupal development of *Drosophila*

We used *Drosophila* fly line carrying a transgene (Dipt2.2-*lacZ*) in which *lacZ* reporter gene is placed under the control of 2.2 kb of diptericin promoter sequence (Reichhart et al., 1992). Diptericin is an inducible antibacterial peptide initially characterized in the fly *Phormia terranovae* (Dimarcq et al., 1988), which is active against Gram negative bacteria. The Dipt2.2-*lacZ*:1 fly line has already been described in detail (Reichhart et al., 1992): the transgene is expressed in a tissue-specific manner, mainly in the fat body. The level of induction is identical to that of the resident diptericin gene at the end of the third larval instar, but lower in adults. We have now measured the kinetics of *lacZ* induction upon bacterial challenge at various stages of development, namely in wandering third instar larvae, and in day 0 (0–3 h), day 1 (24–27 h), day 2 (48–51 h), day 3 (76–79 h) and day 4 (90–93 h) pupae. Adult emergence occurred during day 5. Exposure of wandering larvae to bacteria resulted in a rapid and strong induction that reached maximal values in 6 to 10 h (Fig. 2). The injury sometimes delayed pupariation, but by 24 h all the animals had pupariated. During the first hours of the pupal stage, the transgene was still inducible at levels comparable to those obtained with late larvae, but during pupal development the inducibility decreased markedly, and by day 4 it was no longer possible to induce the transgene. In young adults (0–24 h post emergence), the inducibility of the transgene was restored (data not shown).

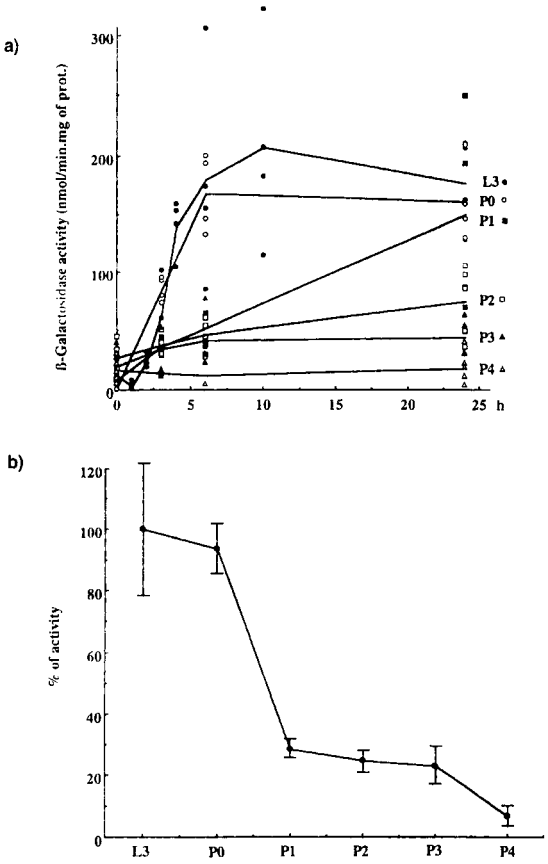

Fig. 2. Inducibility of a diptericin reporter gene in a transgenic *Drosophila* fly line. The animals were challenged with bacteria at different stages as indicated (L3 – wandering third instar larvae; P – pupae) and the β-galactosidase activity was measured at various time intervals after challenge. Each point represents activity in a pool of 5 animals. a) Kinetics of induction of the reporter *lacZ* gene (0–24 h). Each line represents mean quantity of 3 to 5 independent measurements. b) β-Galactosidase activity 6h after bacterial challenge vs age (expressed as a percentage of activity in challenged L3). The data are presented as mean ± SE.

Effect of exogenous ecdysteroids on inducible antibacterial activity in diapausing larvae of *Calliphora*

The influence of 20-hydroxyecdysone (20E) was tested in diapausing *Calliphora* larvae where endogenous ecdysteroid titers are very low (Richard & Saunders, 1987). 20E was injected simultaneously with heat-killed *E. coli* and 2 µl of hemolymph were collected after overnight exposure. After injection, control larvae were maintained at 20°C to follow the dose-dependent morphogenetic effects recorded as average time intervals between ecdysteroid injection and pupariation at 20°C (Fig. 3). The hemolymph samples were tested for their anti-Gram-positive and anti-Gram-negative activity. Pupae challenged by injection of bacteria in the absence of 20E showed a level of activity comparable to that of bacteria-challenged early pupae (see above). The co-injection of increasing amounts of 20E resulted in a decrease in the antibacterial response. This decrease was dose-dependent, but the response could not be abolished totally: only one larva out of 16 which had been bacteria-challenged in the presence of 0.5 µg of 20E exhibited no antibacterial activity.

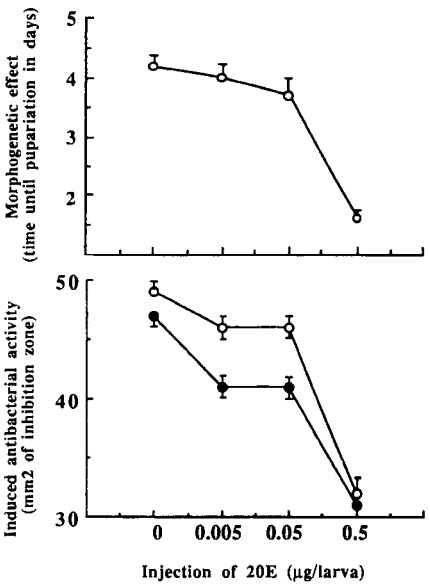

Fig. 3. Effects of injection of 20-hydroxyecdysone (20E) on the immune response of diapausing larvae of *Calliphora vicina*. Increasing amounts of 20E were injected simultaneously with bacteria. Morphogenetic effects were recorded as time intervals between injection and pupariation. Antibacterial activities are given as in Fig. 1. The data are presented as mean ± SE.

Deprivation of the neuroendocrine system in diapausing *Calliphora* larvae: influence on the antibacterial response

Diapausing larvae were divided into 4 groups as indicated in Table 1. The first group was tested for constitutive anti-Gram-negative activity which was found to be above background levels. This result is different from that obtained with pupae which do not exhibit a constitutive antibacterial activity (see above). In the second group, the injection of 10^4 heat-killed bacteria generated a 3-fold increase in the anti-Gram-negative activity. The larvae of the two last groups were ligatured behind the brain and retrocerebral complex before bacterial challenge of the posterior part. The increase of induced anti-*E. coli* activity was similar, whether the pupae had been ligaturated or not. However, the co-injection of 20E with bacteria into the ligatured larvae significantly reduced the antibacterial response in the abdominal fragments.

TABLE 1. Antibacterial activity in normal and neuroendocrine-deprived *Calliphora vicina* larvae. Four experimental groups, each consisting of 20 diapausing larvae, were treated as follows: I – no treatment; II – unligatured larvae injected with 10^4 heat-killed *E. coli* cells in 1 µl; III – larvae ligated behind the brain and retrocerebral complex were injected with 10^4 heat-killed *E. coli* cells in 1 µl; IV – larvae treated as in group III which were in addition injected with a suspension containing 0.1 µg/µl 20E. The larvae were left overnight at 20°C, after which 2-µl hemolymph samples were collected and tested individually for anti-*E. coli* activity.

Experimental group	Size of inhibition zone (in mm^2) mean ± SE	P
I	1 ± 5	
II	38 ± 8	< 0.01
III	34 ± 4	< 0.001
IV	23 ± 2	< 0.05

DISCUSSION

The data presented in this study confirm that dipteran ability respond to bacterial challenge is subject to developmental variation. During the pupal development of *Calliphora*, the highest efficiency for the synthesis of antibacterial molecules is found during the first two days after pupariation. Later, in the period of intense histolysis and histogenesis, the capacity to produce immune peptides after septic injury decreases, and these peptides become almost undetectable by the middle of the pupal stage. This observation is corroborated by a study of the immune response of *Drosophila* in the same period: a transgene consisting of diptericin gene promoter sequences upstream of a reporter *lacZ* coding sequence is strongly inducible at the beginning of the pupal stage and loses its inducibility by the end of this stage.

We show a negative correlation between the immune response of *Calliphora* with the ecdysteroid titres established for the pupal stage of *Calliphora erythrocephala* with *Calliphora* bioassay by Shaaya & Karlson (1965). Since the results of bioassay slightly differ from the radioimmunoassay data on *Calliphora* 20E (Koolman, 1980) this correlation should be verified. However, the same type of correlation is found in *Drosophila* if we consider the composite ecdysteroid titre curve proposed by Richards (1981), which is very similar to the *Calliphora* curve. It is characterized by a sharp peak of hormone titre at puparium formation, and a second maximum reached shortly before the second half of the pupal stage. Experiments performed on diapausing larvae of *Calliphora* showed that a simultaneous injection of 20E and bacteria induces a weaker antibacterial response than the same injection without 20E. Although the effect is modest (2 fold), it indicates that the hormone is directly involved in this phenomenon. This result rules out that the reduced ability to synthesize defense molecules in late pupae is due to depletion of fat body cells or other immune-responsive cells; it is rather in favour of a specific repressive action of the hormone, either direct or indirect, on the expression of defense genes in the pupa.

Isolated *Calliphora* abdomens deprived of the neuroendocrine system produce antibacterial peptides with the same efficiency as intact insects in response to a bacterial challenge. This means that the immune cells do not need the presence of the brain or the ring gland hormones to sustain a normal defense activity. In the same animals, we observed a weak but significant negative regulatory effect of 20E.

Taken together these observations suggest that cross-talk may exist between steroid regulated genes and the insect immune system, reminiscent of glucocorticoid effects on inflammatory mechanisms in vertebrates.

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