The synthesis of antibacterial proteins in isolated fat body from Cecropia silkmoth pupae

Ingrid Faye[1] and G.R. Wyatt

The Group in Eukaryotic Molecular Biology and Evolution, Department of Biology, Queen's University, Kingston (Ontario K7L 3N6, Canada), 14 December 1979

Summary. Fat body from previously immunized diapausing pupae of the silkmoth, *Hyalophora cecropia* (Saturniidae), incubated in vitro, released antibacterial activity into the medium and incorporated ^{3}H-leucine into the immunity proteins P1–P9. The release of antibacterial activity from fat body was also induced to some extent by injection of Ringer solution and, after 20 h in culture, by the injury of dissection.

Pupae of giant silkmoths have a system of humoral immunity which is induced by a primary inoculation with variable, non-pathogenic bacteria or with killed bacteria[2,3]. The induction process was blocked by early injection of actinomycin D or cycloheximide, which indicates a requirement for de novo synthesis of RNA and proteins. The immune proteins constitute too small a part of the total protein content to be observed in normal haemolymph. However, immunity could be induced in diapausing pupae, and during this developmental stage a selective synthesis of 9 new proteins (designated P1–P9) has been demonstrated.

The first step in the induction process is the phagocytosis of the bacteria used as immunizing agent[4]. These were found to become associated with fat body tissue especially in both ends of the pupae. We now report that fat body tissue taken from immunized pupae of *Hyalophora cecropia* produces antibacterial activity and synthesizes P1–P9 during incubation in vitro.

Diapausing pupae of *H. cecropia* were immunized by injection with living *Enterobacter cloacae*. After 3 days, when immunity was well expressed, fat body samples were taken and assayed for ability to synthesize the immune proteins in organ culture. After 5 h of incubation, fat body tissue from an immunized pupa had released significant amounts of antibacterial activity into the medium (figure 1, a). The tissue from either a non-treated pupa or one given an injection with a lepidopteran Ringer solution produced little or no activity. The incorporation of ^{3}H-leucine into total secreted proteins was also stimulated in fat body from an immunized pupa and, to a less extent, in that from a Ringer-injected pupa (figure 1, b).

The immunity proteins P1–P9 can be fractionated by ammonium sulfate precipitations and identified by their electrophoretic mobility in SDS polyacrylamide gel. The

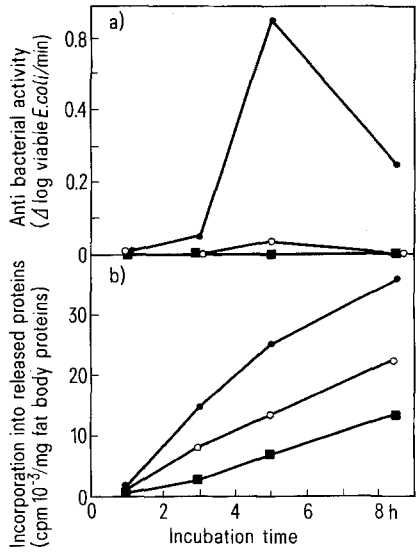

Fig. 1. Antibacterial activity and protein synthesis in fat body cultures of *H. cecropia*. 4 days after injection of *E. cloacae* (strain β12, 5×10^5 viable cells) or lepidopteran Ringer[5] the main part of the fat body tissue from 3 pupae was dissected out and rinsed 3 times in Ringer. The tissue was then incubated at 25 °C in 20 ml of lepidopteran medium[6], containing 100 µM L-leucine and 0.8 µM ^{3}H-leucine (resultant sp. act. 393 mCi/mmole). The medium also contained streptomycin (100 µg/ml). Aliquots of medium were withdrawn at intervals and assayed for antibacterial activity against *Escherichia coli*, strain D31[2] and for radioactive TCA-precipitated protein[7]. At the end of the experiment the fat body tissue was removed from the medium, homogenized and centrifuged. The soluble fat body protein was assayed by the biuret method. The medium was used for further analysis of the proteins (see figure 2). ●, Fat body from immunized pupae (*E. cloacae* injected); ○, fat body from control pupae (Ringer injected); ■, fat body from non-treated pupae.

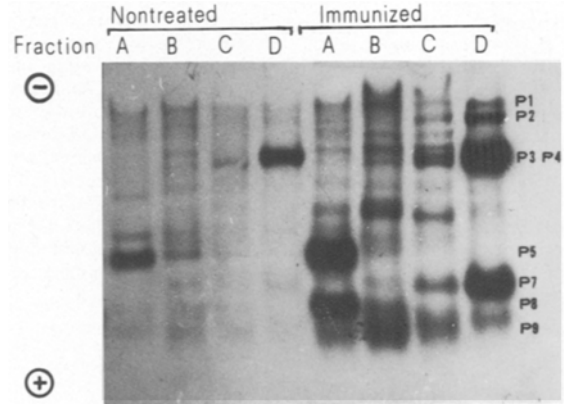

Fig. 2. Polyacrylamide gel electrophoresis of labelled proteins synthesized by fat body tissue in vitro. At the end of an incorporation experiment as described in figure 1, 15 ml medium from fat body cultures of non-treated and immunized pupae were precipitated into four ammonium sulphate fractions; A: 0–38%; B: 38–50%; C: 50–62%; D: 62–75% saturated. The precipitated proteins were dissolved in 500 µl 0.1 M sodium phosphate buffer and 20 µl was run on SDS polyacrylamide gels[8]. The radioactive proteins were visualized by fluorography[9].

proteins present in the culture medium were therefore fractionated by this procedure followed by fluorography. All but 1 (P6) of the 9 known immunoproteins were found to be synthesized by fat body from immunized pupae (figure 2). The distribution between the 4 ammonium sulfate precipitates was the same as found earlier. However, the ratio of the components produced in vitro may not be the same as found earlier in vivo. P7 and P9, which in purified form lyse different bacteria[10] were more abundant than was previously observed. Medium from culturing fat body from a non-treated pupa showed synthesis only of smaller amounts of P5 and P4 (possibly P3). These 2 proteins have been purified[11,12], but their functions are not yet understood.

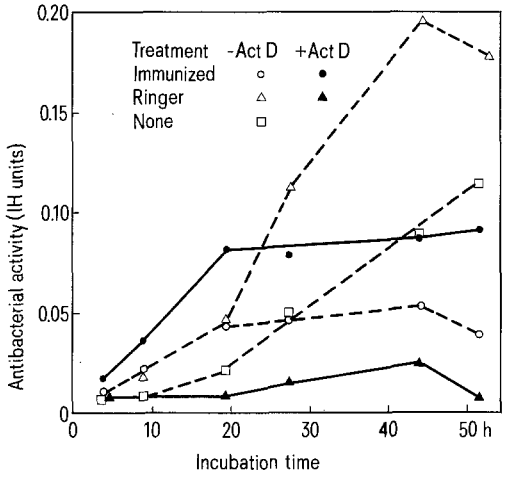

Fig. 3. Effects of actinomycin D on antibacterial activity in cultures of fat body from immunized and Ringer-injected pupae. Pupae were treated and fat body cultures were prepared as in figure 1. Actinomycin D (50 µg/pupa) was injected 3 h before dissection. The medium contained 1 µg of cold leucine per ml. The fat body tissue from each pupa was split into 3 approximately equal parts, each of which was incubated in 2.5 ml medium. At intervals antibacterial activity was assayed by the following inhibition zone technique. 2 µl of medium was applied in circular holes in thin agar plates containing *E. coli* K 12 strain D31 (3×10^3 viable cells/ml). The plates were incubated at 37 °C overnight. The diameters of the clear zones appearing after incubation were measured and related to a standard curve for diluted immune hemolymph. 1 IH unit corresponds to the antibacterial activity in the undiluted hemolymph of an immunized pupa. Each plot represents the mean value of 3 cultures from the same pupa.

It is well known that injuries to insects stimulate metabolic events, including protein synthesis[13-16]. We have also previously shown that an injection of saline solution can induce moderate antibacterial activity[2]. It is therefore reasonable to assume that the effects recorded in figures 1 and 2 with Ringer correspond to an injury reaction. However, since the experiment in figure 1 was of short duration and since the dissection of the fat body in itself could be expected to produce an injury effect, we performed longer incubation experiments using pre-treatment with actinomycin D in order to prevent the response to injury caused by the dissection. Figure 3 shows that fat body from a pupa which had been vaccinated with *E. cloacae* 4 days previously gave immediate, rapid release of antibacterial activity, which levelled off after about 20 h of culture. This release was not inhibited by actinomycin D injected 3 h before dissection. Fat body from pupae given a control injection of Ringer, on the other hand, released activity at a rate that rose steeply after 20 h in vitro and was diminished by actinomycin. In fat body from an untreated pupa there was also a significant release of activity after 20 h.

These observations may be interpreted as follows: 1. An injection of bacteria in vivo programmed in the fat body for synthesis of immunity proteins which was manifested immediately in vitro without requirement for additional mRNA synthesis during the incubation. 2. In pupae injured by injection of Ringer solution, the fat body became programmed for a certain synthesis of antibacterial proteins during the same early period. 3. The dissection itself induced a dramatic antibacterial activity in the fat body which began to be expressed after 20 h in vitro and was dependent on RNA synthesis during the incubation period. 4. That the antibacterial activity in the cultures from vaccinated pupae did not reach the same level as in those from untreated or injured pupae may be due to the presence of living bacteria which may consume the antibacterial factors.

Our results show that antibacterial activity, a function of proteins P1-P9, can be produced by isolated silkmoth fat body from diapausing silkmoth pupae, and that this synthesis can be stimulated by the wounding effects of Ringer injection or dissection, as well as by prior vaccination with bacteria.

1 Present address: Department of Microbiology, University of Stockholm, S-106 91 Stockholm, Sweden. We thank Hans G. Boman for helpful criticism during the preparation of the manuscript. This work was supported by grants from the Swedish Natural Science Research Council, the National Research Council of Canada, and the U.S. National Institutes of Health.

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