Pergamon

PII: S0022-1910(97)00105-4

J. Insect Physiol. Vol. 44, No. 2, pp. 157–164, 1998
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Printed in Great Britain
0022-1910/98 $19.00 + 0.00

The Influence of Bacterial Species and Intensity of Infections on Nodule Formation in Insects

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Received 14 March 1997; revised 19 June 1997

Nodulation is the predominant cellular immune reaction to bacterial infection in insects. Nodulation is a complex process involving an unknown number of discrete cellular actions. Currently, there is only limited information on the signal transduction mechanisms that result in nodulation. In older larvae of the tobacco hornworm, *Manduca sexta*, and of the tenebrionid beetle, *Zophobas atratus*, eicosanoids are involved in one or more steps in the overall process, and treating these insects with inhibitors of eicosanoid biosynthesis prior to bacterial infection severely impairs their ability to form nodules. In this paper we address more detailed questions on eicosanoid-mediated nodulation. The nodulation reaction to bacterial infection occurs in all larval stages we examined, specifically, second, third, and fourth instars of *M. sexta*. In both species, the number of nodules formed in response to bacterial infection is related in an exponential way to the number of bacterial cells in the infection. Nodulation is also not related to larval size. We also found that nodulation intensity varies according to the species of infecting bacteria. **© 1998 Elsevier Science Ltd. All rights reserved**

Manduca sexta *Zophobas atratus* Eicosanoids Cellular immunity Nodulation

INTRODUCTION

Insects elaborate two major defense reactions to bacterial infections, humoral and cellular (Gupta, 1986, 1991). Humoral defense reactions result from regulated biosynthesis of antibacterial proteins, including lysozyme, which enzymatically hydrolyzes bacterial cell walls (Dunn, 1986), and other proteins that act through their detergent properties on bacterial cell membranes (Chernysh *et al.*, 1996). Cellular defense reactions include phagocytosis, carried out mainly by plasmatocytes, and nodulation, which involves granulocytes and plasmatocytes (Gupta, 1991). While the general phenomenology of cellular defense reactions is well appreciated, the signal transduction mechanisms that mediate these reactions remain relatively unknown.

Because many mammalian cellular host defense reactions are mediated by eicosanoids, we suggested that eicosanoids similarly mediate hemocytic immune reactions in insects (Stanley-Samuelson *et al.*, 1991). *Eicosanoid* is a collective term for all biologically active, oxygenated metabolites of arachidonic acid, and two other C20 polyunsaturated fatty acids. The structures and biosynthetic pathways of these compounds are presented in recent reviews (Stanley-Samuelson, 1993, 1994a). In our early experiments, we assessed the influence of eicosanoid-biosynthesis inhibitors on the ability of tobacco hornworms, *Manduca sexta*, to clear artificial bacterial infections from hemolymph circulation. The general finding was that the inhibitors severely impaired the ability to clear bacterial cells from circulation. Whilst we did not speculate on a particular cellular reaction, it appeared that one or more cellular immune responses depended on eicosanoid biosynthesis.

Nodulation (Fig. 1) is the major immediate cellular immune response to bacterial infections (Horohov and Dunn, 1983). Based on our observations of impaired ability to clear bacteria from circulating hemolymph, we hypothesized that nodulation is a specific cellular immune action dependent on eicosanoid biosynthesis (Miller *et al.*, 1994). We found that the relative intensity of nodulation following bacterial infections could be meaningfully assessed in two ways. First, by counting numbers of hemocytic microaggregates under phase contrast optics, or, second, by counting numbers of melanized, mature nodules that appeared within the hemocoel of tobacco hornworms, using a dissecting microscope. Following the protocols developed for our original work,

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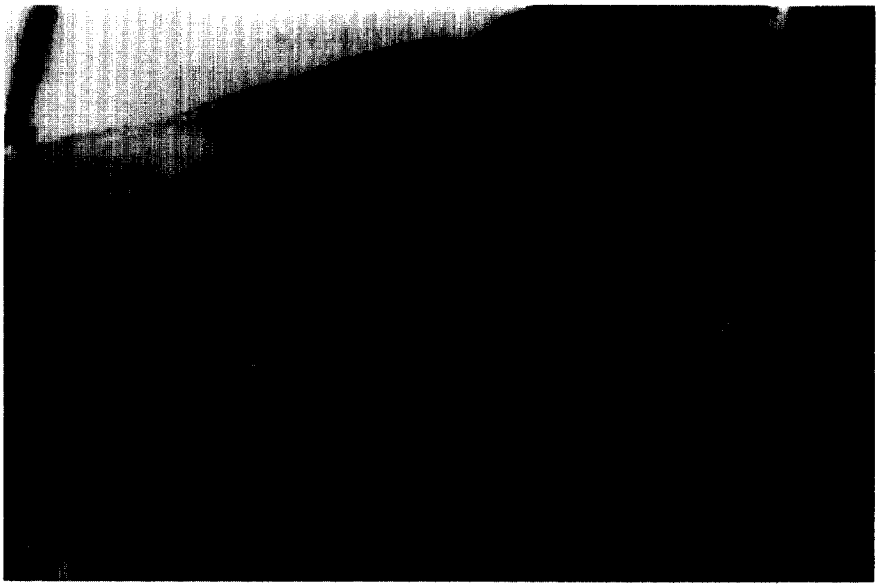

FIGURE 1. Photomicrographs (60 ×) of nodulation reaction to bacterial infection in the beetle *Z. atratus*. Photomicrograph of melanized nodules in response to bacterial infections. The solid arrow points to one of several visible nodules, seen in situ against the background of the larvae fat body. This photograph was taken during routine assessment of nodulation at 6 h PI using an Olympus SZ series stereomicroscope equipped with a SC 35 camera. Photography by J.S. Miller.

we assessed the influence of eicosanoid biosynthesis inhibitors on the ability of hornworms to form microaggregates and nodules in response to bacterial infection. Nodulation was severely reduced in hornworms that had been treated with eicosanoid biosynthesis inhibitors prior to bacterial infection, and we reasoned these results supported the eicosanoid hypothesis (Miller *et al.*, 1994).

The ability to quantitatively assess nodulation in infected tobacco hornworms opens several avenues of inquiry. First, does the nodulation response to bacterial infections depend on eicosanoids in most, if not all, insect species? Second, do eicosanoids act in nodulation during other developmental stages? And, third, can we identify biological factors that influence the intensity of nodulation following bacterial infections? We have addressed the first question by conducting experimentation analogous to our original work, using larvae of several insect species, including the tenebrionid beetle, *Zophobas atratus* (Miller *et al.*, 1996), true armyworms, *Pseudaletia unipuncta*, black cutworms, *Agrotis ipsilon* (Jurenka *et al.*, 1997) and silkworms, *Bombyx mori* (Stanley-Samuelson *et al.*, 1997). The results of these experiments support the idea that eicosanoids mediate nodulation in many insect species.

In this paper we report on additional experiments with *M. sexta* and *Z. atratus* designed to illuminate the remaining questions just posed. We found that the nodulation response to bacterial infections occurs in all larval stages examined and that eicosanoids mediate nodulation in early—as well as in late—stage larvae. We observed that the intensity of nodulation is related to bacterial numbers during infection, and to the species of the infecting bacteria, but is poorly related or unrelated to either insect biomass or body length.

MATERIALS AND METHODS

Organisms

Manduca sexta eggs are provided to our laboratory in weekly shipments from Ms Linda Liska (USDA-ARS, Beltsville, MD). Larvae are reared on standard culture medium in individual cups under semi-sterile conditions developed by Dunn and Drake (1983). To assess the influence of age on nodulation capacity, we used larvae in identified stages.

Larvae of the beetle *Z. atratus* were initially purchased from a local pet store and reproducing cultures have since been maintained in open plastic 'shoe boxes' containing whole wheat flour and rolled oats (10:90, v/v). Water was provided by keeping damp towels on top of the bran. The containers are kept in incubators held in constant darkness at 30°C and approx. 70% r.h.. Because these beetles express indeterminate numbers of larval molts, we identified three categories of larvae for our experiments. These are small ($\leq$ 100 mg), medium ($\leq$ 250 mg), and large ($>$ 1000 mg).

Cultures of a non-pigmented strain of *Serratia marcescens* and nutrient broth (Difco) were purchased from Carolina Biological Supply (Burlington, NC). Cultures of three other species of bacteria, *Escherichia coli*, *Bacillus subtilis*, and *Sarcina flava* were also purchased from Carolina. Bacteria were grown in 50 ml of broth in an environmental shaker at 37°C and 100 r.p.m. For most experiments with *S. marscescens*, bacteria were grown to a titre of 10^7 cfu ml^{-1}, then heat killed in an autoclave. The heat killed bacteria were concentrated and kept under sterile conditions at 4°C. *S. marcescens* was used for all experiments, with the exception of the experiments designed to assess the influence of bacterial species on

nodulation. In these experiments, live bacteria of *S. marscecens* and the three other species were used in the same titres just described.

Injections and nodulation assays

We followed our routine protocols for injecting compounds and bacteria into larvae and for assaying nodulation (Miller *et al.*, 1994; Miller *et al.*, 1996). Test larvae were injected with the PLA_2 inhibitor dexamethasone [(11ß, 16α)-9-fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-dione] (Biomol, Plymouth Meeting, PA). Control larvae were injected with nutrient broth, distilled water, or 95% ethanol. Larvae were surface sterilized by swabbing their surfaces with 95% ethanol. Insects were injected with indicated bacterial dosages in 10 μl aliquots, using a 26 gauge 0.5″ needle attached to a 50 μl syringe (Hamilton, Reno, NV). Drugs and control substances were injected in 2 μl aliquots into the opposite side of the larvae using a 10 μl Hamilton 701 syringe. For second and third instars, the same concentrations of dexamethasone and bacterial cells were used, with injection volumes were adjusted to 1 μl. There was no larval mortality due to injections.

Nodulation (Fig. 1) was assessed at selected times post injection (PI). Larvae were anesthetized by chilling on ice, then the hemocoels were exposed. Melanized dark nodules were counted using a stereomicroscope at 60 ×. The nodules were distinct, and direct counting accurately reflected the intensity of nodulation response (Miller *et al.*, 1994, 1996). In particular, small bits of melanization at the sites of injection were clearly distinguished from nodules. After the initial counting, the alimentary canal was excised, and nodules in the previously unexposed areas and remaining internal tissues were then counted.

Time-course of nodulation

Individuals in six groups of *Z. atratus* larvae (experimentals, water-treated controls and uninjected controls for small and medium larvae) were injected with 10^5 cfu/larva of heat killed bacteria. At 1, 4, 8, and 24 h PI, sub-groups of larvae were anesthetized, and nodulation was assessed.

The influence of bacterial species on nodulation intensity

Similar experiments were conducted for each of four species of bacteria, using general protocols just described. The influence of bacterial species on nodulation was separately assessed for *Z. atratus* and *M. sexta*. Standard dosages of each bacterial species were injected into individuals in four groups of larvae. A control group received a bacterial dose plus an injection of ethanol. One experimental group received a bacterial dose plus an injection of dexamethasone. One experimental group received an injection of dexamethasone, then a bacterial dose, then an injection of arachidonic acid. A second control group was treated as just described, except the final injection was ethanol, the carrier vehicle for the arachidonic acid. We used standard dosages of dexamethasone (Miller *et al.*, 1994, 1996).

RESULTS

Time-course of nodule formation

Fig. 2 shows the time course of visible nodule formation in small and medium sized *Z. atratus* larvae. Both groups of larvae exhibited similar kinetics of nodulation, with two apparent rates of nodulation. At 1 h PI larvae contained fewer than 1 nodule/larva, which increased to about 7–9 nodules/larva by 18 h PI. The number of nodules increased by about 3-fold between 18 and 24 h PI Virtually no nodules were recorded in untreated control larvae at any time during the 24 h experiments. The water treatments, meant to control for the effects of the bacteria carrier vehicle, resulted in significantly (Student's *t*-test, $P < 0.001$) fewer nodules than bacteria treated larvae in both groups of larvae.

Nodulation depends on eicosanoids in second, third, and fourth instar M. sexta larvae

Unlike *Z. atratus* larvae, *M. sexta* larvae undergo a determinate number of molts, and each instar can be definitively identified. We used *M. sexta* larvae to ask if nodulation depends on eicosanoid biosynthesis in second, third, and fourth stage larvae. Table 1 displays the number of nodules formed in response to injections of 10^5 cfu/larvae in second, third, and fourth stage *M. sexta* larvae. Third instars produced about 17 nodules/larva, considerably less than their fourth stage counterparts, which produced 23 nodules/larva.Compared to ethanol-treated control larvae, nodulation in dexamethasone treated larvae was reduced by about one half in third and fourth stage larvae.

We also investigated nodulation in second stage tobacco hornworms. Second stage larvae were able to form nodules, however, the nodules were much smaller than the nodules usually observed in larger hornworms. Moreover, nodulation in second stage larvae was attended by substantial tissue necrosis. Hence, the number of nodules

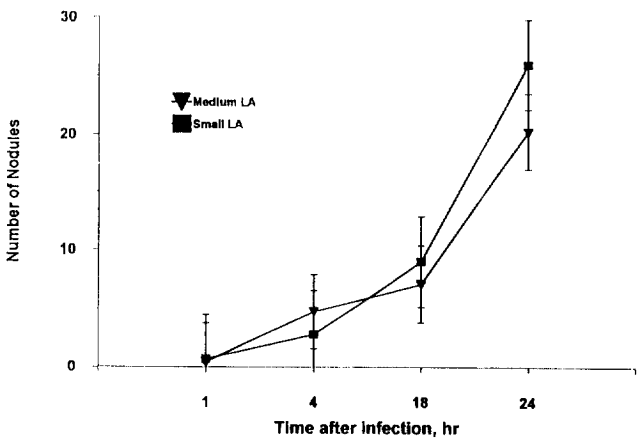

FIGURE 2. Time-course for nodulation in small and medium sized larvae of the beetle *Z. atratus*. Larvae were subjected to intrahemocoelic injections with heat killed cells of the pathogenic bacterium *S. marcescens*. Nodulation was assessed at the indicated times PI. Each point represents the mean number of nodules found in each larva (number of larvae) and the error bars represent 1 SEM.

TABLE 1. The eicosanoid biosynthesis inhibitor, dexamethasone, impaired nodulation reactions to bacterial infections in second, third, and fourth stage tobacco hornworms, *M. sexta*

Larval stage	Ethanol-treated control larvae	Dexamethasone-treated experimental larvae
2nd instar, day 1*	55.2 ± 14.7 (5)	36.9 ± 8.7 (9)
3rd instar, day 1	17.3 ± 4.9 (6)	8.9 ± 1.9 (11)
4th instar, day 2	23.0 ± 3.8 (5)	12.8 ± 2.2 (10)

* Nodules in second instars were much smaller and more numerous compared to larger larvae

Experimental insects in each stage were injected with dexamethasone dissolved in ethanol and control insects were injected with ethanol. Larvae in both groups were then injected with 10^5 cfu of heat-killed bacteria. At 4 h PI, the number of nodules formed in response to bacteria was assessed. The reported values represent mean number of nodules, ± 1 SEM. (n) = number of individual larvae. Means for experimental larvae are statistically different from means for control larvae at $P < 0.005$ (Student's t-test).

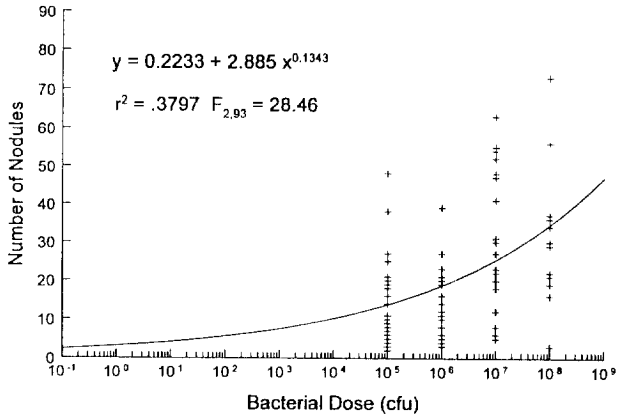

FIGURE 4. Nodulation relates to the size of bacterial infections in a dose-dependent manner in medium larvae of the beetle *Z. atratus*. Larvae were subjected to intrahemocoelic injections with the indicated number of heat killed cells of the pathogenic bacterium *S. marcescens*. Nodulation was assessed at 4 h PI. Each point represents the number of nodules found in individual larvae and the solid line represents the exponential relationship shown on the figure.

seen in second stage larvae is not comparable to the number of nodules seen in third, fourth, and fifth stage larvae. Nonetheless, nodulation in second stage larvae appears to be mediated by eicosanoids because dexamethasone-treated second instars yielded considerably fewer nodules than their ethanol-treated counterparts (Table 1).

The influence of bacterial dosage on nodulation intensity

Nodulation intensity, as measured by numbers of nodules at 4 h p.i., was tightly related to bacterial dosages in *M. sexta* and *Z. atratus* (Figs 3 and 4). Fig. 3 shows that nodulation in fifth instar *M. sexta* declined from 142 nodules/larva at 10^6 cfu/infection to about 25 nodules/larva at 10^2 cfu/infection. A similar pattern emerged from experiments with medium and large size *Z. atratus* larvae (Fig. 4). In large size larvae nodulation declined from a high of about 31 nodules/larva at 10^7 and 10^8 cfu/infection to 14 nodules/larva at 10^5 cfu/larva (Fig. 4). Figs 3 and 4 also show that bacterial dosages and nodulation responses were related in an exponential way for both species. In the medium size larvae, nodulation declined from about 25 nodules/larva at 10^5 cfu/infection to about 8 nodules/larva at 10^4 cfu/infection (data not shown). Although this trend is in line with results from large larvae, we did not attempt to fit a curve to this narrow range of bacterial dosages.

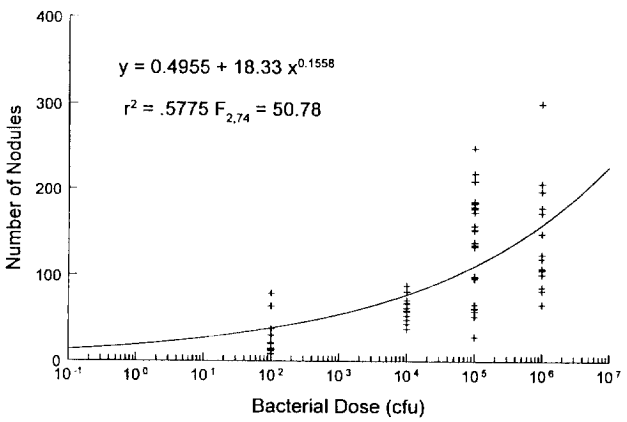

FIGURE 3. Nodulation relates to the size of bacterial infections in a dose-dependent manner in *M. sexta*. Larvae were subjected to intrahemocoelic injections with the indicated number of heat killed cells of the pathogenic bacterium *S. marcescens*. Nodulation was assessed at 6 h PI. Each point represents the number of nodules found in individual larvae and the solid line represents the exponential relationship shown on the figure.

Nodulation is not strongly related to larval size

The relationship between larval size, as expressed by mass, and nodule formation in response to a standard dosage of bacterial cells is shown in Fig. 5. These data indicate that for *M. sexta* (Fig. 5), nodulation is not correlated to larval weight within a single instar ($R^2 = 0.27$). We obtained similar data for *Z. atratus*. We also determined that another parameter of larval size, length, is not

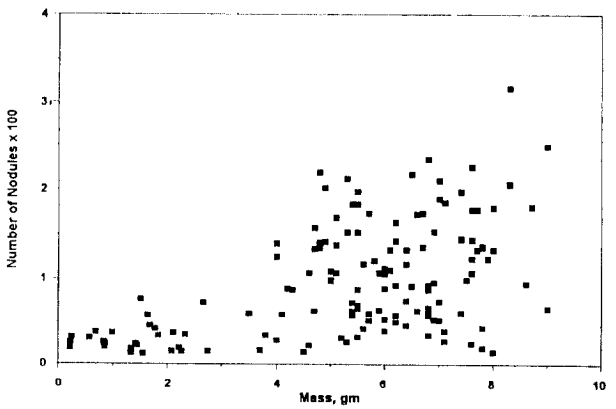

FIGURE 5. Nodulation is not related to insect size, as measured by mass, in *M. sexta*. Larvae were injected with a standard dosage of heat killed cells of the pathogenic bacterium *S. marcescens*. Nodulation was assessed at 4 h PI. Each point represents the number of nodules found in each larva.

related to nodulation formation in response to a standard dosage of bacterial cells (data not shown).

The influence of bacterial species on nodulation intensity

The influence of bacterial species on nodulation intensity is shown for *M. sexta* (Fig. 6) and for *Z. atratus* (Fig. 7). The overall patterns are similar for both species. *S. marcescens* and *S. flava* induced high levels of nodulation, over 80 nodules/larva in *M. sexta* and nearly 60 nodules/larva in *Z. atratus*. *Escherichia coli* induced lower levels of nodulation, about 40 nodules/larva in *M. sexta*. *Bacillus subtilis* induced quite low levels of nodulation, approx. 20 nodules/larva in *M. sexta* and in *Z. atratus*.

Figs 6 and 7 also show that the nodulation reactions to all four bacterial species were reduced in larvae that had been treated with dexamethasone. Moreover, the dexamethasone effects were reversed by treating the dexamethasone-injected larvae with arachidonic acid.

DISCUSSION

Here, we document the relationship between dosages of injected bacteria and intensity of nodulation reactions, and show that nodulation depends on eicosanoid biosynthesis in all stages of larval development examined. These data indicate that nodulation is a carefully metered, graded response to bacterial infections.

Nodulation is the first and quantitatively major cellular reaction to bacterial infections in *M. sexta*, and probably most insects (Horonov and Dunn, 1983). The process (Fig. 1) begins with formation of about 5–20 hemocytes, termed microaggregates, which trap bacterial cells. The microaggregates increase in size by continued recruit-

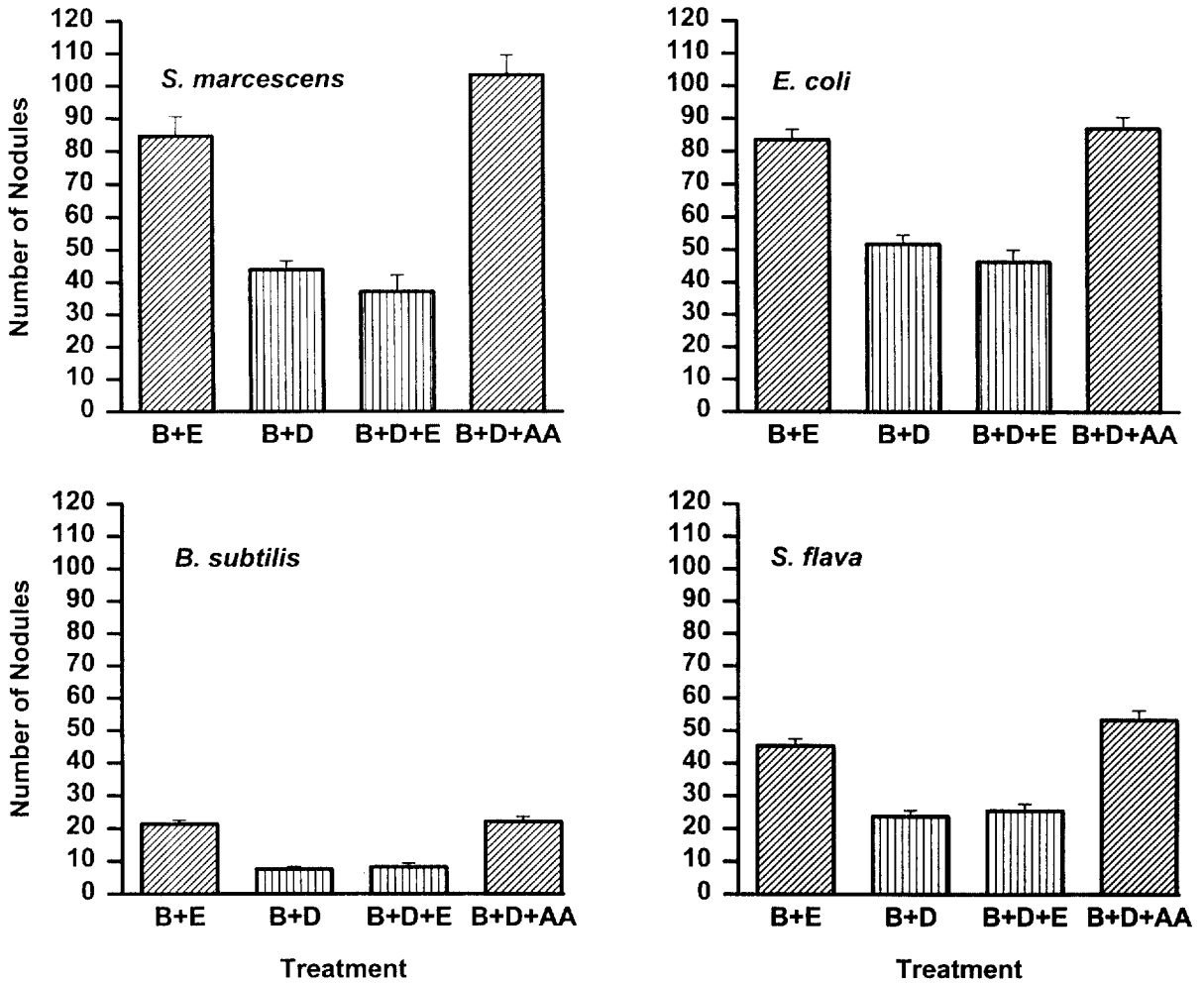

FIGURE 6. The influence of standard dosages of four bacterial species (indicated on each panel) on nodulation reactions to bacterial infections in fifth stage *M. sexta* larvae. For each bacterial species, four groups of larvae were treated. Individuals in group 1 (B + E) were injected with ethanol, then with a single bacterial species. Individuals in group 2 (B + D) were injected with dexamethasone dissolved in ethanol, then with a single bacterial species. Individuals in group 3 (B + D + E) were injected with dexamethasone dissolved in ethanol, then with a single bacterial species. Immediately after the bacterial injections, larvae were injected with ethanol. Individuals in group 4 (B + D + AA) were injected with dexamethasone dissolved in ethanol, then with a single bacterial species. Immediately after the bacterial injections, larvae were injected with arachidonic acid. The histogram bars represent mean number of nodules, and the error bars indicate 1 SEM. Histogram bars with the same fill pattern are not significantly different from each other (LSD, $P < 0.05$).

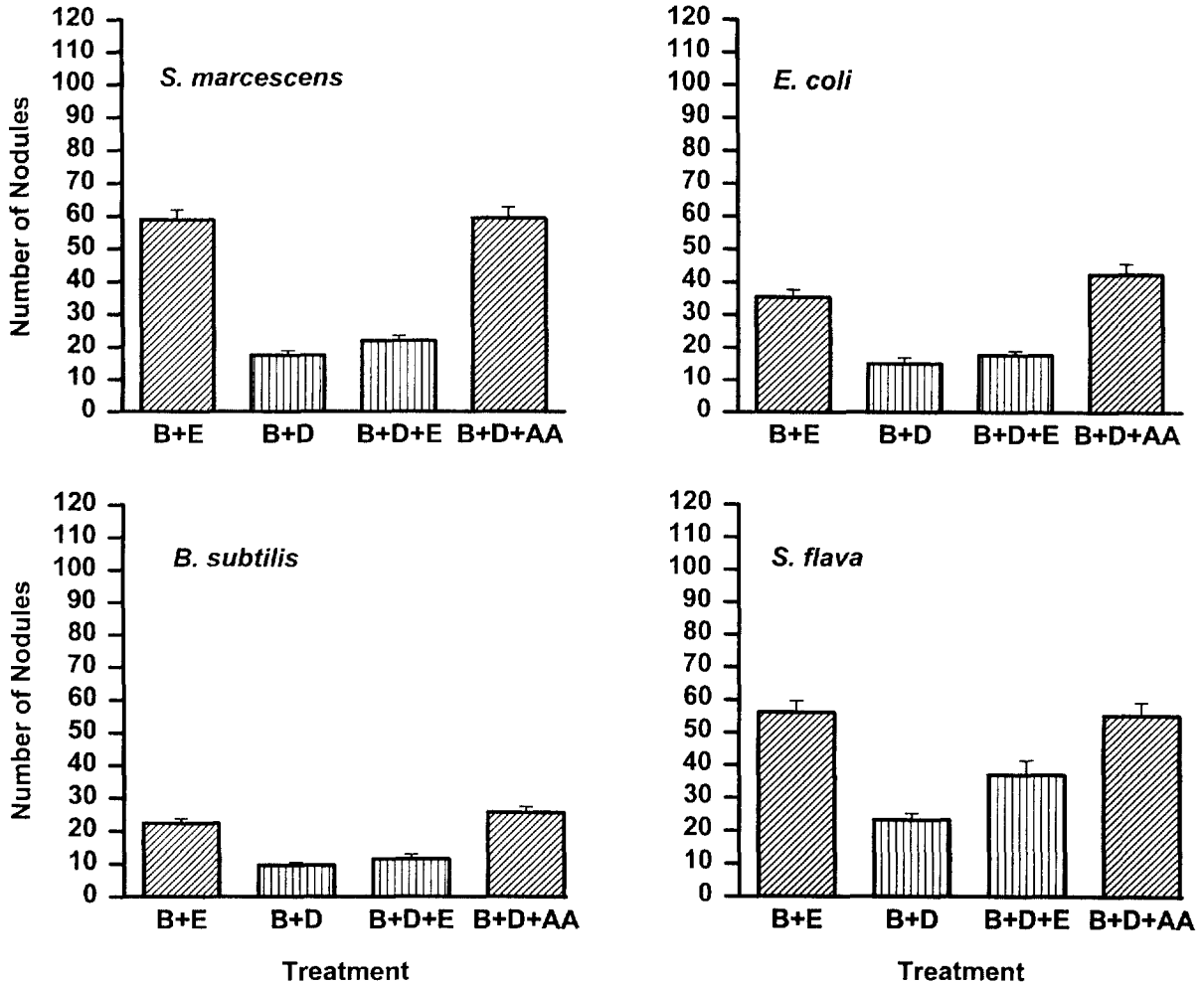

FIGURE 7. The influence of standard dosages of four bacterial species on nodulation reactions to bacterial infections in fifth stage *Z. atratus* larvae. Four groups of larvae were treated. Individuals in group 1 (B + E) were injected with ethanol, then with a single bacterial species. Individuals in group 2 (B + D) were injected with dexamethasone dissolved in ethanol, then with a single bacterial species. Individuals in group 3 (B + D + E) were injected with dexamethasone dissolved in ethanol, then with a single bacterial species. Immediately after the bacterial injections, larvae were injected with ethanol. Individuals in group 4 (B + D + AA) were injected with dexamethasone dissolved in ethanol, then with a single bacterial species. Immediately after the bacterial injections, larvae were injected with arachidonic acid. The histogram bars represent mean number of nodules, and the error bars indicate 1 SEM. Histogram bars with the same fill pattern are not significantly different from each other (LSD, $P < 0.05$).

ment and addition of hemocytes and bacterial cells. The overall process ends with formation of melanized nodules containing hemocytes, bacteria and some hemocytes which have phagocytosed bacterial cells. Although we know very little of the detailed cellular actions that result in nodulation, appreciation of the general steps in the process suggests that numerous cellular signal transduction steps are involved in the regulated initiation, growth, and maturation of nodules.

The intensity of insect nodulation reactions is related to two categories of factors: those intrinsic to the nature of the invading bacteria and those intrinsic to the insect. With respect to the bacteria, Ratcliffe and Walters (1983) reported that pathogenic strains of bacteria resulted in greater levels of nodulation than did non-pathogenic strains in the greater waxmoth, *Galleria mellonella*. Rahmet-Alla and Rowley (1989) came to a similar conclusion in their work with the Madeira cockroach, *Leucophaea maderae*. Beyond this, in some cases, the pathogenicity of bacteria may be related to the ability of bacteria to interrupt cellular defense reactions. For example, Ratcliffe and Gagen (1976) found that larvae of *G. mellonella* and *Pieris brassicae* were competent to form nodules in response to several nonpathogenic bacteria, but nodules were not formed in reaction to the pathogen *Staphylococcus aureus*. Finally, the intensity of nodulation seems to depend on features of the interaction between living bacteria and their insect hosts. Rahmet-Alla and Rowley (1989) found that the differences in cellular defense reactions observed in response to living bacteria did not occur in response to similar dosages of heat-killed bacteria. Our findings accord with the general idea that the nature of infecting bacteria influences the nodulation response. We note high levels of nodulation

in response to two bacterial species (*S. marcescens* and *S. flava*), and considerably lower levels of nodulation in response to two other bacterial species (*B. subtillis* and *E. coli*). That the extent of nodulation is related to bacterial species indicates the insects are able to measure certain features of invading bacterial cells, and develop appropriately scaled hemocytic responses.

We have shown that *M. sexta* and *Z. atratus* larvae are competent to calibrate nodulation reactions to the sizes of bacterial infections. We found in medium and large *Z. atratus* larvae, as well as fifth instar *M. sexta*, that as more bacterial cells are injected, more nodules are formed. Such a relationship seems intuitive; however, the nature of the relationship has not been considered. Although one might expect a linear relationship between number of infecting bacterial cells and the number of nodules formed in reaction to the infection, we found otherwise. These parameters are appropriately related by exponential functions. We make sense of it in this way: while the number of bacterial cells increases in a linear way, the total bacterial surface area increases exponentially. We infer that the calibration of defense reactions is based on features of bacterial surfaces; the greater the surface, the more response.

In large *Z. atratus* larvae, nodulation increased with increasing bacterial dosages to about 30 nodules/larva. We examined the effects of up to 10^8 bacterial cells, and conclude that this insect's capacity to form nodules is approx. 30 nodules/larva. Nodulation is similarly related to increasing bacterial dosages in *M. sexta* larvae. *M. sexta* larvae exhibited a considerably greater capacity to form nodules, reaching an upper limit of about 300 nodules/larva. The nodules observed in both species were discreet formations of about the same size, and we conclude that counting nodules is an appropriate measure of nodulation intensity. Because *Z. atratus* and *M. sexta* exhibited considerably different capacities to form nodules in response to similar bacterial challenges, we considered the possibility that nodulation is related to the size of the insects. However, size *per se*, as determined by larval mass or by larval length, was found not to be a predictor of capacity to form nodules. This apparent paradox can be resolved by considering the influence of size on nodulation within a particular size range. This is most clearly illustrated by results with *M. sexta* (Fig. 5). While larvae weighing about 1 gm generally produced fewer nodules than larvae weighing about 7 gm, some 8 gm larvae produced fewer nodules than their 1 gm counterparts. We surmise that the intrinsic capacity to form nodules in response to similar challenges is related to other features of the larvae, perhaps to the number of available circulating and sessile hemocytes.

As previously established for mature *Z. atratus* and *M. sexta* larvae (Miller *et al.*, 1994, 1996), our data show that nodulation depends on eicosanoid biosynthesis in all developmental stages we examined. The significance of this work relates to cellular signaling systems involved in nodulation. The first experiments were conducted on *M. sexta*, from which we suggested eicosanoids mediate nodulation in most, if not all, insect species. As recounted in the introduction, we have been working to test this idea by working on additional insect species. Again, all of the original work was done on fifth instars, for the convenience of working with relatively large insects. It would be reasonable to suppose eicosanoids similarly act in nodulation in all larval stages competent to produce nodules in response to bacterial infections. In these experiments, we tested this idea. We probed the eicosanoid biosynthetic pathways by treating the larvae with dexamethasone, which inhibits all eicosanoid biosynthesis through its influence on phospholipase A_2 activity. Nodulation was severely reduced in the experimentally treated larvae. While this approach is not without conceptual difficulties (Stanley-Samuelson, 1994b), we have established control experiments that allow us reasonably to infer that eicosanoid biosynthesis is an essential step in cellular immune signaling.

We also note, as seen in the original work (Miller *et al.*, 1994, 1996), inhibition of eicosanoid biosynthesis did not completely ablate the ability to form nodules. We take these findings to indicate that other signaling moieties are involved in cellular immune reactions. Biogenic amines are good candidates, and indeed, Downer and his colleagues have shown that these moieties are involved in hemocytic reactions, including phagocytosis, nodulation and cellular movements (Baines *et al.*, 1992; Baines and Downer, 1994; Diehl-Jones *et al.*, 1996). While hemocytic activities are often discussed in broad terms, actions such as phagocytosis and nodulation are made up of many (still unknown) individual cellular events. It is not difficult to imagine that eicosanoids, amines and other, still unidentified, signal moieties are essential elements in the overall cellular defense processes.

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Acknowledgements—We thank Drs Leon Higley, Brenda Oppert and James Peterson for useful comments on a draft of this paper. Dr Higley also performed the mathematical treatments, for which we are very grateful, indeed. Thanks to Wyatt Hoback for help with the figures. This is paper no. 11528, Nebraska Agricultural Research Division, and contribution no. 921 of the Department of Entomology. This article reports the results of research only. Mention of a proprietary product does not constitute an endorsement or recommendation for its use by the USDA. This work was supported by the Agricultural Research Division, UNL and by USDA-ARS Specific Cooperative Agreement # 58-5430-5-115.