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Insect Immunity

I. Characteristics of an Inducible Cell-Free Antibacterial Reaction in Hemolymph of *Samia cynthia* Pupae

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Pupae of the silk moth, *Samia cynthia*, were found to contain an inducible antibacterial activity in their hemolymph. This immunity response was provoked by primary infections with either *Escherichia coli* K-12 or *Enterobacter cloacae*. In both cases the antibacterial activity was directed chiefly towards *E. coli*. During standard conditions, 1% of hemolymph could kill 10^3 to 10^4 viable *E. coli*, strain D31, within 5 min. A lower level of antibacterial activity was induced by injections of a sterile salt solution. The killing of strain D31 followed single-hit kinetics, and increasing rate constants were obtained for increasing amounts of hemolymph. The reaction was sensitive to pretreatment with trypsin and it was protected by reducing agents. The activity was inhibited by microgram quantities of lipopolysaccharide (LPS) prepared from certain LPS mutants of *E. coli* K-12. A comparison of the susceptibility showed that "heptose-less" LPS mutants were more sensitive to killing than other strains. During standard conditions hemolymph will lyse both *E. coli* and *Micrococcus lysodeikticus*. Lysis of *E. coli* followed a multi-hit kinetics and it was inhibited by LPS, whereas lysis of *M. lysodeikticus* was unaffected by LPS. Hemolymph was fractionated on Sephadex G-200, and the lytic activities were recovered in partly overlapping peaks. Reconstitution with pooled fractions gave synergistic effects with the killing assay.

Estimates indicate that the number of insect species may be close to a million and that the total insect population of the world may be as large as 10^{18} (38). For this reason alone, insect immunity is a problem in its own right. In addition, studies of insect immunity may be motivated by the facts that: (i) insects are known to lack immunoglobulins and organ transplantations are unopposed by defense mechanisms like those in mammals (4, 9, 17); (ii) insects are known to transmit helminths, protozoa, bacteria, and viruses which cause severe human diseases (19); and (iii) microbial control of pests is currently tried as an alternative to chemical insecticides (8, 26). For each of these aspects, knowledge of insect immunity would further both understanding and applications.

Despite an extensive early literature (9, 17, 33), relatively little is known about the molecular mechanism behind immunity phenomena in insects. Within the last decade, some authors have studied the antibacterial activity of insect hemolymph (2, 10, 11, 15, 18, 21, 24, 27). Here we characterize an inducible antibacterial activity, which develops in pupae of the silk moth *Samia cynthia* after primary infections with either *Escherichia coli* or *Enterobacter cloacae*. These bacteria are known to be associated with other silk moths and with the wax moth (33). Larvae of the latter animal have also been used for several studies of immunity (10, 11, 18, 21, 24, 27, 31, 36). Pupae of large *Lepidoptera* seem to have received little or no attention, which is odd since the very stable diapause of animals like *S. cynthia* make them excellent for laboratory studies. In addition, the molting of the gut may in some species make the immune system of special importance in pupae (31).

MATERIALS AND METHODS

Bacteria. The main test organism used was *E. coli* K-12, strain D31, which carries the amino acid markers given in Table 1 as well as λ prophage. The strain is resistant to streptomycin and to ampicillin due to mutations in the *strA* and *ampA* genes, respectively. In addition, D31 carries two less-known mutations, one closely linked to *ampA*, and one which deleted part of the glucose, galactose, and rhamnose from the core of the lipopolysaccharide (LPS) (25). Together they enhance the ampicillin resistance on

TABLE 1. *Origin, requirements, and antibiotic response of the main bacterial strains used*

Organism	Strain	Amino acid requirements	Antibiotic response		Reference or origin
			Growth on	No growth on	
E. coli K-12[a]	D31	Pro, Trp, His	80 Amp; 100 Str	30 Nal; 20 Kan	(4)
E. cloacae	β11	None	100 Str; 10 Pen	80 Amp; 30 Nal	Materials and Methods
E. cloacae	β12	None	30 Nal; 10 Pen	100 Str; 80 Amp	Materials and Methods
P. aeruginosa	OT97	His, Trp	20 Kan; 100 Str	80 Amp; 30 Nal	From Loutit
B. thuringiensis	Bt11	Ser, Thr, Asp, Ile, His, Val, Met, Glu	100 Str	80 Amp; 10 Pen	Materials and Methods

[a] Other *E. coli* strains used are also resistant to streptomycin and sensitive to Nal and Kan. They are related to D31 as described elsewhere (5, 7). For selective growth on LA plates the respective antibiotic was added to the final concentration in micrograms per milliliter as indicated by numbers before the abbreviation. Except for Bt11 two alternative selections are given and abbreviations are as follows: D-ampicillin (Amp); streptomycin (Str); benzyl-penicillin (Pen); nalidixic acid (Nal); kanamycin (Kan). Amino acids are abbreviated as follows: aspartic acid (Asp); glutamic acid (Glu); histidine (His); isoleucine (Ile); methionine (Met); proline (Pro); serine (Ser); threonine (Thr); tryptophan (Trp); valine (Val).

plates to 80 μg/ml. Besides D31 we used also some other related LPS mutants for which pedigrees and other details are given in two reviews (5, 7). (These strains have been deposited at the *E. coli* Genetic Stock Center, Department of Microbiology, Yale University School of Medicine, New Haven, Conn., where they can be requested.) For the present experiments LPS was prepared by the method of Galanos et al. (13).

The other bacteria used (Table 1) were obtained as follows. The parental strain β1 of *E. cloacae* was isolated from a pile of waste bark near Umeå. The mutants β11 and β12 were isolated on LA plates (25) with streptomycin or nalidixic acid, respectively. The parental strain of *Bacillus thuringiensis* var. *gelechiae* AUCT is a non-crystal-forming strain obtained from Sebasta (32) but originating from Institute Pasteur. From the parent we isolated a clone able to grow on a glucose salt medium supplemented with the eight amino acids given in Table 1. This clone was used for the isolation of a streptomycin-resistant mutant designated Bt11. *Bacillus subtilis* was a wild-type strain kept in our stock collection.

All strains were grown exponentially in a rich medium to a cell density of about 4×10^8 cells per ml by using conditions described before (25). The bacteria were harvested by filtration, washed twice on the filter with 0.9% NaCl, and resuspended in NaCl to about the same cell concentration (without any chilling). The bacteria were used as soon as possible after harvest; just before an assay they were diluted 50 to 100 times in the standard buffer (see below). Except for *B. subtilis* the bacterial strains used were resistant to one or more antibiotics, properties which were used for selective plating with the antibiotic concentrations given in Table 1. This procedure eliminates the low level of bacterial contamination which could come from our semisterile standard assay (see below).

Standard assay for antibacterial activity. Unless noted otherwise, the standard buffer was 0.1 M sodium or potassium phosphate, pH 6.4, with 2×10^{-3} M dithiothreitol (DTT, "Cleland's reagent," Sigma Chemical Co., St. Louis, Mo.). The final reaction mixture contained normally 5 or 10 μliters of 10 to 20-times-diluted *S. cynthia* hemolymph and 100 μliters of test bacteria (normally strain D31) diluted in the standard buffer to give a final concentration of about 5×10^5 cells per 0.1 ml. (Reactions were carried out in 1.5-ml conical tubes of polypropylene, carefully washed and reused.) Using disposable capillary pipettes ("Microcaps," Drummond Scientific Co., Broomall, Pa.), 5-μliter samples were withdrawn from the reaction mixture at different times and emptied into 1 ml of ice-cold 0.9% NaCl. This dilution and chilling effectively stopped the killing reaction. The samples were further diluted, transferred to 3 ml of soft agar at 45 C, and spread on LA plates which for strain D31 were supplemented with 80 μg of ampicillin or streptomycin per ml at a concentration of 100 μg/ml (25). After about 30 min a second layer of soft agar was spread on each plate, a procedure which allowed an accurate counting of up to 1000 colonies per plate. Incubation conditions for plates were 15 to 18 h at 37 C.

Lysis of bacteria. The substrate was either freshly grown *E. coli* strain D31 or dried *Micrococcus lysodeikticus* obtained from Mann Research Laboratories (catalogue no. 935). The cells were suspended in the standard buffer, giving an absorbance of 0.3 to 0.5 at 570 nm. Lysis was followed in 1-ml samples by using continuous recording with a Beckman double-beam spectrophotometer, Acta CII. The reference contained 1 ml of the same cell suspension. The hemolymph added to the reaction mixture had a significant absorption at 450 nm. At this wave length we therefore added an equal volume of hemolymph from untreated pupae to the reference cell.

Insects. Most experiments were performed with chilled diapausing female pupae of *S. cynthia* obtained from C. M. Williams, Harvard University, but originating from American dealers. One batch of pupae was raised in Umeå on the synthetic diet recommended by Riddiford (28). The larvae were reared under short-day conditions (10L:14D) to provoke pupael diapause (C. M. Williams and L. M. Riddiford, personal communication). Temperature

was always around 25 C and humidity was 60 to 80%. The weight of the American female pupae was 2 to 3 g, whereas those raised in Umeå were only half the size. Pupae in diapause were stored in a refrigerator at 8 C for at least 10 weeks. During experiments all animals were kept under long-day conditions (15L:9D) at 25 C and 60 to 80% humidity to terminate the diapause and cause initiation of adult development.

Small samples of *S. cynthia* hemolymph (5 to 25 μliters) were taken up in capillaries directly from incisions (2 to 4 mm) in the wings. Exhaustive bleedings were done as recommended by Schneiderman (30) by using a few crystals of 1-phenyl-2-thiourea (Eastman Kodak Co., Rochester, N.Y.) to inhibit the phenolase. Such samples were centrifuged at 500 × *g* for 15 min before use.

Vaccination of insects. Pupae of *S. cynthia* were normally vaccinated by injecting into the thorax 4×10^5 *E. coli*, strain D31, or in a few experiments *E. cloacae* strains β11 or β12. An "AGLA" micrometer syringe (Wellcome Reagents Ltd., London) was used and the volume was always 50 μliters. Control animals were given an equal dose of "W-saline," a salt mixture physiological to *Lepidoptera* (35) (made up from four parts of NaCl, 40 parts of KCl, 18 parts of $MgCl_2$, and three parts of $CaCl_2$, the molarity of each of the solutions being 0.15 M). Bacteria used for injection were diluted in W-saline, and care was taken that each animal in an experiment was given an equal number of injections. At least 2 days were allowed for immunity to develop.

RESULTS

Induction of S. cynthia antibacterial activity. For *Drosophila* we have previously shown that a primary infection (referred to as "vaccination") with *E. cloacae* induced a defense system which protected the flies against a secondary infection with *Pseudomonas aeruginosa*, an organism which otherwise would kill the flies (6). To explore the biochemistry behind this protection it was important to reproduce the *Drosophila* experiment with a larger insect. We therefore took pupae of *S. cynthia*, injected one group with *E. cloacae* (dose of about 3×10^6 viable bacteria per pupa) and the other group with a similar volume of W-saline. Four days later all pupae were challenged by an injection of *P. aeruginosa* (dose of about 10^6 viable bacteria per pupa). Figure 1 shows that this secondary infection was rapidly eliminated from the hemolymph of pupae vaccinated with *E. cloacae*, whereas in the control pupae *P. aeruginosa* grew up to more than 10^9 cells per ml of hemolymph. We also followed the concentration of *E. cloacae* in the hemolymph, and Fig. 1 shows that the primary infection ("vaccine") persisted longer than *P. aeruginosa*. This is in agreement with our earlier findings (6) and indicates that for reasons not yet known,

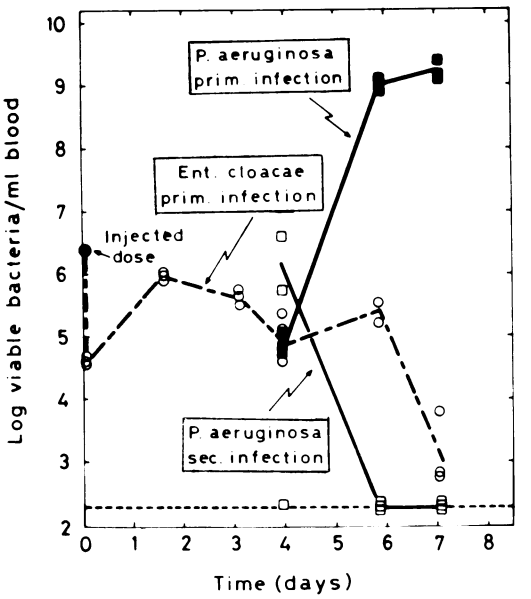

FIG. 1. *Vaccination of S. cynthia pupae with E. cloacae and test for immunity by a secondary infection with P. aeruginosa. At the start of the experiment three pupae were injected with E. cloacae* (○), *three control pupae were given W-saline. On day 4 all pupae were given injections with about* 10^6 *viable P. aeruginosa (control pupae,* ■; *vaccinated pupae,* □). *At the times indicated 5-μliter samples of hemolymph were withdrawn from the pupae and assayed for viable bacteria by plating on media containing the selective antibiotics given in Table 1. Each individual pupa is represented by one point and the dotted line in bottom part of the figure indicates the lowest level of bacteria that could be detected.*

the primary infecting organism became less susceptible to host defense than was the strain used for secondary infection.

Similar experiments have shown that the antibacterial activity in the hemolymph could be induced also by infections with *E. coli* strain D31 despite the fact that the organism was rapidly eliminated from the hemolymph. Induction of immunity could occur in developing pupae of both sexes as well as in diapausing pupae. However, no significant immunization was found with living *P. aeruginosa* or *B. thuringiensis*, which both gave fatal infections.

In vitro assay of antibacterial activity with E. coli strain D31. As test organism we selected *E. coli* strain D31, an ampicillin- and streptomycin-resistant LPS mutant, hoping that our previous knowledge of the strain (5, 7, 25) might be helpful for understanding the antibacterial activity. Our aim was to find conditions under which in vitro killing of strain D31 could be used as an assay. Preliminary experiments showed

that reactions performed at room temperature and pH 6.4 with reducing agents like DTT required only 1% hemolymph to give 99% killing in less than 5 min. By using the procedure given in details in Materials and Methods we compared the antibacterial activity of pupae treated in different ways.

Significant antibacterial activities were induced both by living bacteria (*E. coli* strain D31) and by a sterile physiological salt solution (W-saline) (Fig. 2). The response obtained with bacteria was always larger and lasted longer than the corresponding effect of W-saline. Immune responses with salt solutions have been reported for other systems (15, 24) and can be explained as wound effects created by the injections. This interpretation is consistant with the observation that in vivo W-saline gave no protection against infections (cf. Fig. 1). However, further experiments are needed to investigate whether the difference in Fig. 2 is only quantitative or also qualitative.

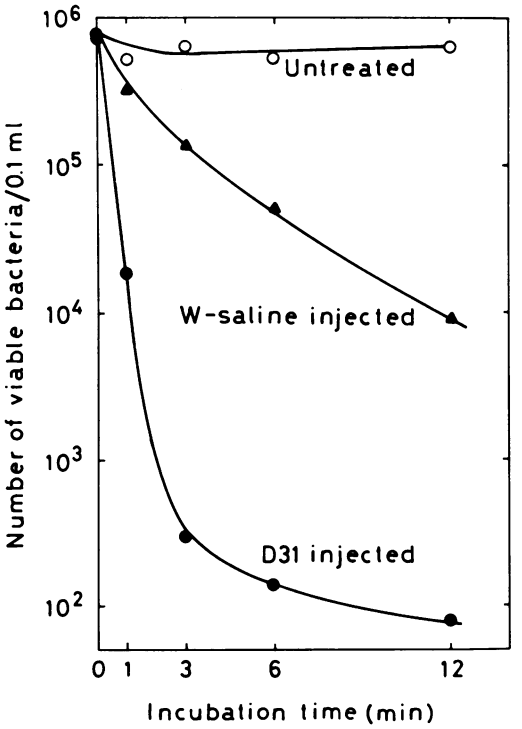

FIG. 2. *In vitro assay of the antibacterial activity of 1% of hemolymph from S. cynthia. The test organism was E. coli, strain D31, and the reaction conditions are given in detail in Materials and Methods. Symbols: pupa vaccinated with living E. coli strain D31, ●; pupa injected with an equal volume of sterile W-saline, ▲; untreated pupa, ○.*

The left part of Fig. 3 shows that at the onset of the reaction the rate of killing followed an apparent single-hit kinetics. However, with low concentrations of hemolymph the rate of the reaction decreased with increasing times. With increasing amounts of hemolymph in the reaction mixture there was an increase in the killing rate. Assuming single-hit kinetics, we have in the right part of Fig. 3 plotted the rate constants obtained from the data in the left part versus the amount of hemolymph in the reaction mixture. With an initial concentration of 3.4×10^5 viable bacteria per 0.1 ml, there was a near-linear relationship between the reaction constants, although the line does not extrapolate to zero.

Properties of the antibacterial reaction. The assay for antibacterial activity uses as "substrate" whole living bacteria. Although the reaction may involve one or more insect enzymes, the sensitivity of the "substrate" makes it impossible to use many of the biochemical reagents frequently employed for the characterization of enzymatic reactions. However, in order to optimize the sensitivity of the assay and possibly to discriminate between different reactions in a multicomponent system, we investigated the factors listed in Table 2. The results indicate that the defense system was noncellular, that at least one component was lost during a dialysis, that a reducing agent like DTT offered some protection, and that at least one component was sensitive to pretreatment with trypsin. A comparison of LPS obtained from three interrelated strains showed that LPS types, which have lost parts of the core polysaccharide (25), were better inhibitors than intact LPS from wild-type *E. coli* K-12.

Species and strain specificities of the killing reaction. Mammalian immunity is characterized by a narrow specificity in the defense mechanism induced. Although previous investigators have emphasized that insect immunity does not have a similar specificity (9, 17), referees of an earlier manuscript demanded that we investigate this point in detail. We have therefore compared the killing of four different bacteria by using 5% of hemolymph from two different pupae, one vaccinated with *E. coli* strain D31 and the other with *E. cloacae* strain β12. Figure 4 shows that in both of the pupae the antibacterial activity was directed primarily against *E. coli*. The killing of the three other bacteria was hardly significant. However, we know from in vivo experiments like that in Fig. 1 that both *P. aeruginosa* and *E. cloacae* are eliminated in vaccinated pupae. Since the ratio between hemolymph and injected bacteria is

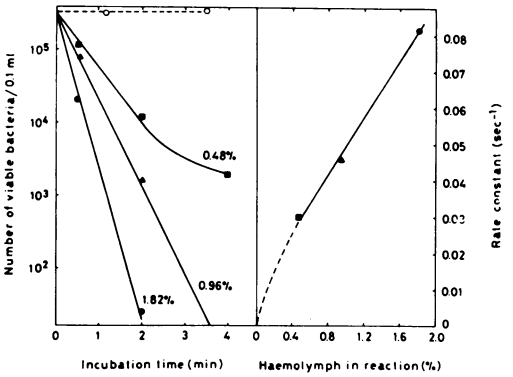

FIG. 3. *Antibacterial activity obtained with different concentrations of hemolymph from pupa vaccinated with living E. coli strain D31. Left, Number of viable bacteria (strain D31) at different times; right, calculated rate constants versus final concentration of hemolymph in reaction mixture. For the reaction with 0.96% hemolymph the point at 4 min is not shown. A control with bacteria and no hemolymph is included in the left part (open circles).*

vastly different in vivo, we performed an experiment with 95% hemolymph. During such in vitro conditions both *P. aeruginosa* and *E. cloacae* were slowly killed. However, further investigations are required to determine whether the humoral immunity here studied can fully account for the in vivo protection against *P. aeruginosa*.

The only gram-positive bacterium included in Fig. 4, *B. thuringiensis*, was neither killed in vitro nor in vivo. We have therefore also tested *B. subtilis* and found that with 5% of hemolymph there was 95% killing within 5 min. This reaction was unaffected by LPS from strain D31.

Table 2 shows that the inhibitory power of LPS was affected by the carbohydrate composition of the polysaccharide part. We have therefore compared a number of our LPS mutants (7, 25) with respect to their susceptibility to killing by 1% of hemolymph (Fig. 5). Together with other results it indicates that our *E. coli* K-12 strains could be classified as follows. The least susceptible group contained strains with a normal LPS, the parental strain D21, as well as our *envA* mutant D22, which is highly permeable to a number of antibiotics (5). A group of moderately sensitive strains, which have lost some carbohydrates in their LPS, included our common test organism, strain D31 as well as the *lpsA* mutant D21e7. The most susceptible group contained our two "heptose-less" mutants, D31m4 and D21f2, both of which have lost all heptose, glucose, galactose, and rhamnose in their LPS (7, 25). We have recently shown that LPS mutants have defects in the barrier function of their outer membranes (7). The difference illustrated in Fig. 5 could therefore be due to an LPS dependent difference in the uptake of the antibacterial activity.

Lytic action of hemolymph. Earlier investigators have reported lytic activities both in insect hemolymph (10, 15, 24, 27) and in the gut (39). We therefore investigated whether hemolymph from *S. cynthia* pupae vaccinated with *E. coli*, strain D31, could also lyse strain D31 and *M. lysodeikticus*. In both cases we found lytic action. With *E. coli* and low concentrations of hemolymph, there was a lag period before any detectible lysis occurred (Fig. 6). This is consistent with a multi-hit kinetics and with the interpretation that a number of chemical bonds have to be split before the bacteria start lysing.

The lysis of *E. coli* did not normally go to completeness. The addition of more hemolymph (indicated by the arrows) reinitiated both the killing and the lytic reactions (Fig. 7).

TABLE 2. *Properties of S. cynthia antibacterial activity*

Treatment of hemolymph	Relative killing of strain D31[a]	
	After 1 min	After 5 min
Centrifugation (600 g/10 min)	1	1
Dialysis (4 h)	1	4×10^{-3}
Preincubation without DTT (45 min)	8×10^{-2}	7×10^{-2}
Pretreatment with trypsin (15 min)	1×10^{-2}	1×10^{-3}
Trypsin added after 1 min		0.2
D21 LPS (1 μg/ml)	0.3	0.2
D31 LPS (1 μg/ml)	0.2	6×10^{-2}
D31m4 LPS (1 μg/ml)	0.2	6×10^{-2}

[a] Relative killing was defined as the ratio between the number of viable bacteria in the control and the number of viable bacteria in the treated sample using standard assay conditions. All viable count values were interpolated from time curves as in Fig. 2. Dialysis was against water with 2×10^{-3} M DTT. In the pretreatment experiment trypsin concentration in the reaction was 100 μg/ml; when added after 1 min it was 1 mg/ml. In the latter experiment relative killing after 1.5 min was 1. LPS was added to the hemolymph 15 min before the start of the reaction. LPS from strain D21 contains in the outer part of the core polysaccharide, heptose, glucose, galactose, and rhamnose. D31 LPS has lost part of the glucose, galactose, and rhamnose. D31m4 is a "heptose-less" mutant with an LPS lacking heptose, glucose, galactose, and rhamnose (25).

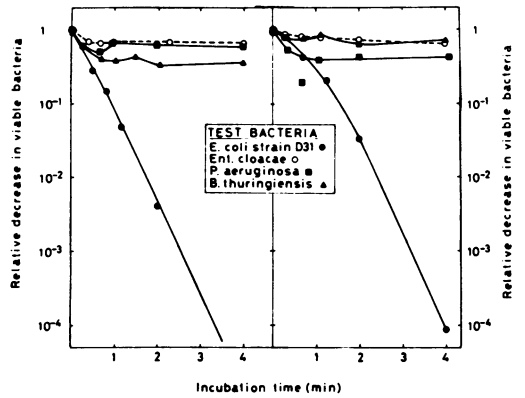

FIG. 4. *Susceptibility of different bacteria to the antibacterial activity in hemolymph (5%) from pupae vaccinated with E. coli strain D31 (left) and E. cloacae strain β11 (right). The test organisms were E. coli strain D31 (●), E. cloacae strain β11 (○), P. aeruginosa strain OT97 (■), and B. thuringiensis strain Bt11 (▲).*

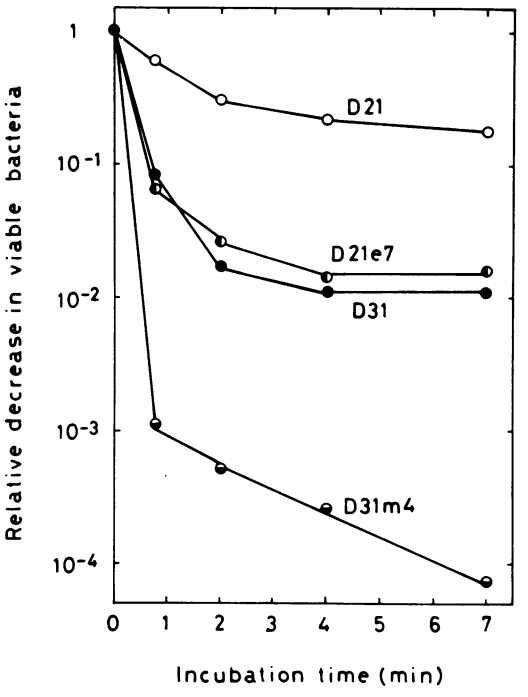

FIG. 5. *Comparison of the susceptibility of different LPS mutants of E. coli K-12 to the antibacterial activity of 1% of S. cynthia hemolymph. Strain D21 (○) has LPS with an outer core polysaccharide containing heptose, glucose, galactose, and rhamnose; strain D31 (●) has lost part of its glucose, rhamnose, and galactose; strain D21e7 (◐) has lost part of its glucose and all of its galactose and rhamnose; strain D31m4 (◒) has lost all its heptose, glucose, galactose, and rhamnose.*

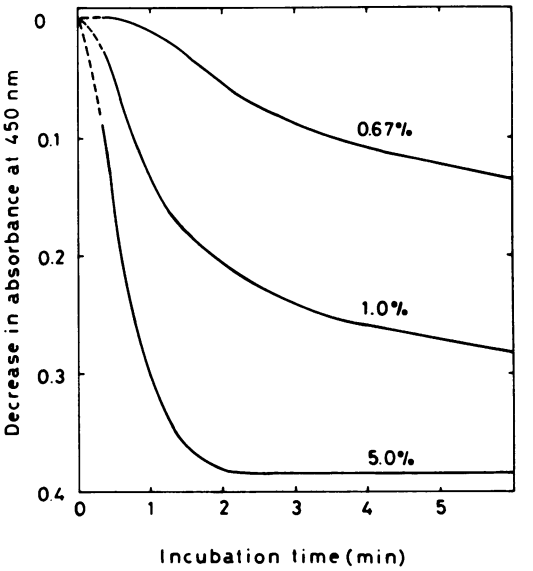

FIG. 6. *A continuous recording of the lysis of E. coli strain D31 with hemolymph in the concentrations indicated in the figure. Reactions were carried out in a Beckman spectrophotometer, Acta CII (27 to 28 C) by using the same conditions as for the killing reaction. The initial concentration of bacteria was about 2 × 10⁷ cells per ml, which gave an extinction of 0.430. The reference was a similar bacterial suspension to which was added hemolymph from an untreated pupa.*

This pattern of kinetics is consistent with either a rate-limiting component being consumed or with product inhibition of an enzymatic reaction. The killing reaction proceeded faster than the lysis and there was no activity in an untreated pupa (Fig. 7).

Since the lytic action can be expected to set free significant amounts of LPS, we examined the reaction for LPS inhibition. Lysis of *E. coli* was susceptible to LPS inhibition, whereas lysis of *M. lysodeikticus* was insensitive to the same high concentration of LPS (Fig. 8). The results imply that the lyses of the two bacteria were due to separate enzymes.

Gross fractionation of S. cynthia hemolymph. If the antibacterial activity in hemolymph were due to a multicomponent system, it would ultimately have to be separated and characterized by reconstitution experiments. Should the complexity be large, one might expect separation on ion exchanger to give loss of activity. On the other hand, some activity could be expected after a moderately resolving fractionation by gel filtration. So far these predictions have come true. We lost almost all activity during separation attempts

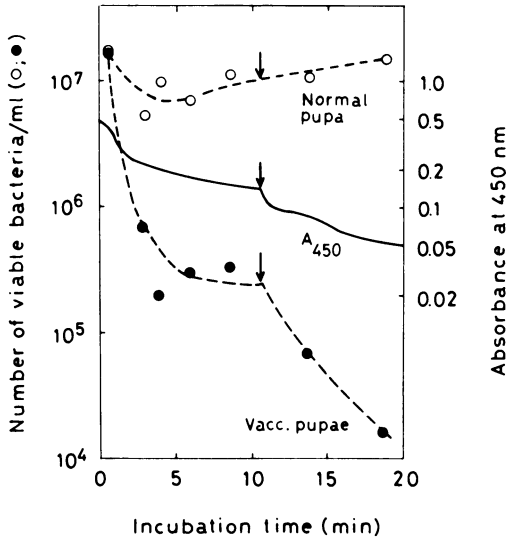

FIG. 7. *Simultaneous recording of lysis (solid line) and the killing (broken line) of E. coli strain D31, using 1% of hemolymph from a pupa vaccinated with strain D31 (●) and from a control pupa without any pretreatment (○). After 10 min (as indicated by the arrows), a fresh sample of hemolymph was added to the reaction mixtures. In this experiment, the samples withdrawn were 1 μliter (in other experiments 5 μliters), which increased the pipetting errors. Reference and other conditions were as in Fig. 6.*

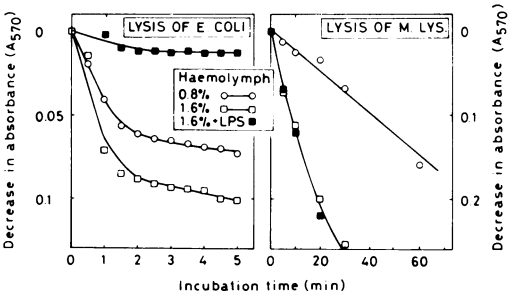

FIG. 8. *Lysis of E. coli (left) and of M. lysodeikticus (right) at two different concentrations of hemolymph (0.8%, ○; 1.6%, □). To the higher concentration was added LPS from strain D31 (■) to a final concentration of 50 μg/ml. The reference used contained only bacteria.*

on anion or cation exchangers with cellulose skeletons. However, Sephadex G-200 resolved hemolymph into two main peaks, which were well separated (Fig. 9). In an intermediary zone, we could identify the lysing activities against *E. coli* and *M. lysodeikticus*, respectively.

In comparable fractionations we assayed also for the killing of strain D31. For those experiments the eluant from the same column as in Fig. 9 was pooled approximately as follows. Sample A with the yellow pigment of the hemolymph was 20 to 40 ml; sample B was 41 to 57 ml; sample C with most of the antibacterial activity was 58 to 68 ml; sample D was 69 to 77 ml; and sample E was 78 to 93 ml. With these fractions we made several reconstitution experiments. A synergistic effect could be demonstrated by combining samples B and E (Fig. 10). Fraction A had neither any activity alone nor in combination with other fractions. Different combinations with the other fractions gave in most cases some synergistic effects, but the magnitude fluctuated from experiment to experiment. These variations could have been due both to difficulties in making reproducible cuts in the eluate and to a marked instability of the isolated fractions.

DISCUSSION

Is the antibacterial activity of insect origin? Up to now the following experiments imply that the activity studied was produced by the pupae and not the living bacteria used as vaccine: (i) *E. coli* strain D31 or our *E. cloacae* mutants can be used either as vaccine or as test organism in vivo (6); (ii) no killing was observed when these strains used were grown in mixed cultures; (iii) when injected into pupae, *E. coli* strain D31 was rapidly eliminated from the hemolymph. However, pretreatment of the pupae with actinomycin D allowed strain D31 to grow up in the hemolymph (manuscript in preparation). This drug does not affect *E. coli* since it cannot normally penetrate the outer membrane of gram-negative bacteria. Since in the pupae actinomycin D will block RNA syn-

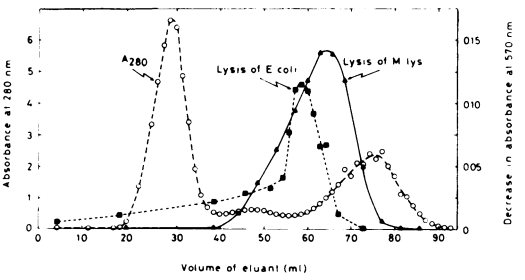

FIG. 9. *Fractionation of 1.0 ml of S. cynthia hemolymph obtained from two pupae vaccinated with E. coli strain D31. The column (44 by 1.35 cm) with Sephadex G-200 (Pharmacia, Uppsala, Sweden) was equilibrated with 0.1 M potassium phosphate buffer, pH 6.4, with 2 × 10^{-3} M β-mercaptoethanol. The eluant from the column was assayed for absorbance at 280 nm (○) for lysis of E. coli strain D31 (■, and for lysis of M. lysodeikticus (▲). Both determinations of bacterial lyses were made after incubation of 0.1 ml of sample and 1 ml of cell suspension at 37 C for 45 min.*

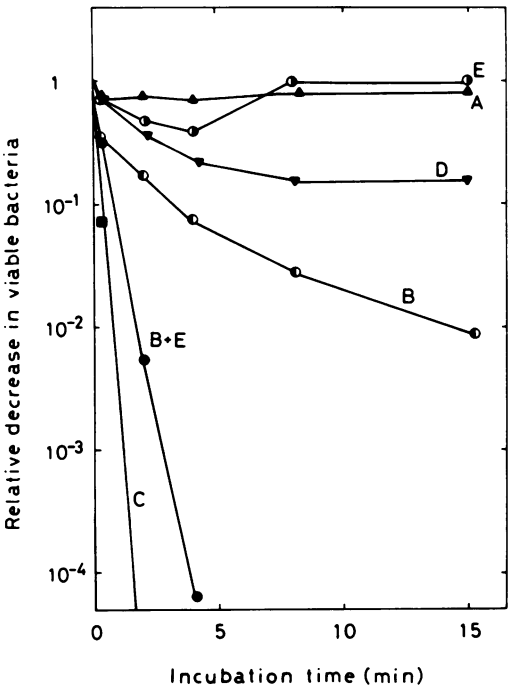

FIG. 10. *Antibacterial activity against E. coli strain D31 in single fractions (A, ▲; B, ◐; C, ■; D, ▼; E, ◑) and the combined fractions B+E (●), all obtained from a column experiment similar to that shown in Fig. 9. The fractions were pooled as given in the text. For C there was an additional point not shown.*

thesis, the expression of immunity must require this biosynthetic activity. Especially the last type of experiment makes it very likely that the antibacterial factors here studied were of insect origin.

S. cynthia system and the assays used. In pupae of some insect species, the main function of the immunity may be to defend the animal against invasions of the natural flora, which could occur during moltings. Especially large risks may come from transitions of the digestive system (37). If so, this would explain both the lack of specificity and the fact that the antibacterial activity here recorded may be significantly higher than previously reported for larvae of *Drosophila* (2, 3) or *Galleria mellonella* (9, 18).

For several reasons it is very difficult to compare our results with others. Earlier investigators have rarely documented that their antibacterial assays give proportionality and their results can therefore not be evaluated quantitatively. Our Fig. 3 shows that for the killing of strain D31 there was proportionality with 1 to 2% of hemolymph. Lysis as recorded in Fig. 6 to 8 is only suitable as a semiquantitative assay but has the advantages of being fast and convenient. Our results with different strains (Fig. 4 and 5) clearly show that comparative results can be obtained only if the same bacterial strain is used as test organism. The fact that strain D31 is well characterized (5) together with its high susceptibility makes it a suitable test organism. We suggest that it would be a useful standard reference for comparing results obtained in different laboratories.

We have so far assayed immunity in more than 100 individual pupae of *S. cynthia*, treated in a variety of ways. In vaccinated pupae we could normally predict the killing of strain D31 by a factor of 2 to 10. This reproducibility may be satisfactory considering the fact that the animals are not inbred. We have encountered certain problems in storing hemolymph and sometimes lost most of the activity even during a rapid freezing. Despite these complications the high antibacterial activity found in vaccinated pupae of *S. cynthia* makes them a very good material for the study of insect immunity.

Mechanism of the bacterial killing. Earlier literature contains different judgements about the role of agglutination and phagocytosis in insects (15, 16, 18, 29, 36), and a hemagglutinating protein has been isolated from a marine invertebrate (23). By using hemolymph from vaccinated pupae, we have so far not detected any microscopic evidence for agglutination. Table 2 shows that a large dose of trypsin (1 mg/ml) added after 1 min only gave a small decrease in the relative killing after 5 min. There was no detectible reversing of the killing reaction. These experiments therefore make it unlikely that agglutination contributes to the rate of killing observed.

The insect lysozymes induced by heat-killed bacteria (27) may either be part of the defense against gram-positive bacteria or function as chitinases in the repair of the exoskeleton (22). The fact that gram-negative bacteria normally are resistant to egg-white lysozyme makes it unlikely that closely related enzymes (27) could be the only factor responsible for the killing of *E. coli*. Moreover, we have shown that the killing and lysis of *E. coli* was inhibited by LPS, whereas the activity against *B. subtilis* and *M. lysodeikticus* (Table 2 and Fig. 8) was unaffected by LPS. It is therefore unlikely that lysozymes of the type purified from *Bombyx mori* and *G. mellonella* (27) can fully account for the immunity in *S. cynthia*.

In all our killing experiments with strain D31, we initially observed single-hit kinetics (Fig. 2 and 3). This is contrary to the lysis of strain

D31, where low hemolymph concentrations gave a lag (Fig. 6) compatible with a multi-hit mechanism. We have so far been unable to separate the killing and the lytic activities against strain D31, and the lysis could therefore be either a late stage of a repeated killing reaction or a late step in a sequence of reactions leading to the elimination of the bacteria.

Sense of the LPS inhibition. We have here shown two effects of LPS on the antibacterial defense system of *S. cynthia*. (i) When hemolymph was preincubated with LPS with a reduced core polysaccharide, there was a pronounced inhibition of the bacterial killing (Table 2). (ii) Bacterial strains with such LPS were more susceptible to killing than the parental strain with wild-type LPS (Fig. 5). Both of these effects are consistent with the hypothesis that one or more components of the defense system have a strong affinity for either lipid A or the innermost core of the LPS molecule.

From a biological point of view it may look senseless for a host to have a defense system inhibited by LPS. However, Andersson et al. (1) have shown that LPS in vitro can induce the synthesis of immunoglobulins in mammalian lymphocytes. Chadwick and Vilk (11) have reported that large doses of crude LPS in wax moth larvae can induce an immunity lasting about 48 h. In mammals LPS is also known to have an anticomplementary activity (14), and this fact has been used for tracing complement-like systems in some in vertebrates (12, 20). Although some of our results could indicate that *S. cynthia* has a complement-like system, more data are required to justify such a conclusion.

We have sometimes encountered difficulties in reproducing Chadwick and Vilk's immunization with LPS (11) by using *S. cynthia* pupae and our own LPS samples prepared by the method of Galanos et al. (13). Disregarding factors like possible species differences (21), the discrepancy could be resolved by one or more of the following assumptions. (i) The active signal in Chadwick and Vilk's experiments was either an impurity or a degradation product of LPS. (ii) As suggested for mammals (34), two signals may be needed and LPS (or lipid A) may be only one of the signals. (iii) The molecule inhibited by LPS could be part of a feedback signal which turns on the gene system needed. The last assumption is in consonance with the observation that a low dose of LPS (0.1 μg) in wax moth larvae could induce a large increase in hemocytes (31). Clearly, separation and characterization of the components of the hemolymph as well as an effective immunization with "dead" material are required for a better understanding of the antibacterial activity and the LPS effect.

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