Lipopolysaccharides of *Xenorhabdus nematophilus* (Enterobacteriaceae) and Their Haemocyte Toxicity in Non-immune *Galleria mellonella* (Insecta: Lepidoptera) Larvae

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Three varieties of *Xenorhabdus nematophilus* subsp. *nematophilus* released lipopolysaccharide (LPS) during bacteraemia in larval *Galleria mellonella*. Larval serum triggered release of LPS from the bacterial envelope. LPS activated the plasmatocytes and eventually damaged the haemocytes. LPS and its lipid A portion bound to the insect haemocytes through D-glucosamine-binding lectins on the haemocyte surfaces. The toxicity of LPS resides in the fatty acids of the lipid A moiety.

INTRODUCTION

*Xenorhabdus nematophilus*, a mutualistic associate of the entomoparasitic nematode *Steinernema feltiae*, is voided by its nematode vector into the haemolymph of insects which the nematode has entered (Poinar & Himsworth, 1967). Greater wax moth (*Galleria mellonella*) larvae respond to bacteria in the haemolymph by nodulation and phagocytosis mediated by haemocytes called granular cells and plasmatocytes (Ratcliffe & Rowley, 1979). *X. nematophilus* subsp. *nematophilus* var. *dutky* is removed from the *G. mellonella* haemolymph by nodulation after modification of the bacterial cell envelope by the haemolymph (Dunphy & Webster, 1984). *G. mellonella* similarly removes *X. nematophilus* subsp. *nematophilus* var. *mexicanus* from the haemolymph immediately after its injection, and these bacteria damage the granular cells and plasmatocytes by reproducing in or on them, before re-entering the haemolymph (Dunphy & Webster, 1986). The unidentified damaging agent does not require concurrent bacterial metabolism for its release into the haemolymph, which implies that a structural component of the bacterial envelope is probably involved (Dunphy & Webster, 1984). Kamionek (1975) and Seryczynska & Kamionek (1977) showed that lipopolysaccharides from an unidentified subspecies of *X. nematophilus* elevated *G. mellonella* haemocyte counts, and this elevation has been associated with the occurrence of haemocyte damage (Dunphy & Webster, 1984). However, until now there has been no proof of lipopolysaccharide release from *X. nematophilus* in *G. mellonella* haemolymph or of its mode of action.

Toxins produced by Gram-negative insect-pathogenic bacteria *in vitro* are assumed to be factors associated with the virulence of these bacteria *in vivo* (Lysenko, 1976). However, there are few reports of bacterial toxin production *in vivo* in non-immune insects. Ratcliffe & Walters (1983) and Walters & Ratcliffe (1983) attributed the destruction of nodules containing *Bacillus cereus* in the haemocoel of *G. mellonella* larvae to the metabolism of these virulent bacteria. Horohov & Dunn (1984) detected, in the haemolymph of *Manduca sexta* infected with *Pseudomonas aeruginosa* 11-1A, a cytotoxin that induced vacuoles in the haemocytes; but the toxin appeared too late in the infection to be the cause of bacteraemia.

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The objectives of the present study were to identify a haemocytotoxin produced by *X. nematophilus* subsp. *nematophilus* varieties in larval *G. mellonella* and to propose a model for the mode of action of the toxin on larval haemocytes.

**METHODS**

*Cultures and media.* *X. nematophilus* subsp. *nematophilus* vars *dutky*, *breton* and *mexicanus* were isolated from Thimersol (0.1%, w/v; Sigma) surface-sterilized, dauer juvenile stages of the DD136, breton and mexican strains, respectively, of the entomopathogenic nematode *Steinernema feltiae* (Dunphy & Webster, 1984). The bacteria were routinely subcultured at 27 °C on Tergitol-7 agar (Difco) containing 2,3,5-triphenyltetrazolium chloride (30 mg l⁻¹) (Akhurst, 1980).

*Bacillus subtilis* (ATCC 6051) and *Proteus vulgaris* 11-1A were maintained on tryptose soy agar (Difco) at 27 °C and subcultured at 7 d intervals.

For experimental purposes 24 h bacterial cultures were grown in LB-broth (Bertani, 1951) for 24 h at 27 °C on a horizontal, gyrotary shaker (150 r.p.m.). The bacteria were centrifuge-washed (5000 g, 10 min, 10 °C) three times in phosphate-buffered saline (PBS: 137.9 mM-NaCl, 2.7 mM-KCl, 8.1 mM-Na₂HPO₄, 1.5 mM-KC₁, pH 6.5; Dunphy & Webster, 1985).

The release of lipopolysaccharides from the *Xenorhabdus* varieties was monitored *in vitro* in artificial *Galleria* larval serum with (10%, v/v) and without a larval serum supplement (Dunphy & Webster, 1986).

*G. mellonella* larvae were reared until the sixth instar (Dutky et al., 1962).

**Lipopolysaccharide (LPS) release, extraction and fractionation.** LPS released by the three *Xenorhabdus* varieties *in vivo* and *in vitro* was monitored using the *Limulus* amoebocyte lysate assay (Associate of Cape Cod, Inc., Woods Hole, Mass., USA). Serum from *Galleria* larvae infected with *Xenorhabdus* had the bacteria removed by centrifugation (10000 g, 5 min, 10 °C) so that 'infected' serum could be tested for *in vivo* endotoxin release. LPS was extracted from the three lyophilized *Xenorhabdus* varieties and from the infected serum using the phenol/water procedure (Westphal & Jann, 1965), purified by ultracentrifuge-washing (100000 g, 3-5 h, 4 °C) five times with distilled water (Kropinski et al., 1982), lyophilized and stored at −20 °C.

Lipid A was obtained as a precipitate by boiling 10 mg *Xenorhabdus* LPS in 1 ml 1% (v/v) acetic acid for 2 h (Mattsby-Baltzer et al., 1984). The precipitates were centrifuge-washed three times (5000 g, 10 min, 5 °C) in distilled water followed by extraction with chloroform/methanol (5:1, v/v) (Russa et al., 1985).

The water fractions from the initial LPS hydrolysates containing the oligosaccharide portions were centrifuged (100000 g, 4 h, 4 °C) to remove unhydrolysed LPS and lipid A. The supernatants were lyophilized either directly or after exhaustive dialysis against distilled water. A subsequent amoebocyte lysate assay did not detect endotoxin activity in these fractions.

Total fatty acid extracts from the lipid A of each of the three *Xenorhabdus* varieties were obtained by hydrolysing 10 mg of each lipid A in 6 ml 6 M-HCl at 100 °C for 2.5 h followed by alkaline hydrolysis in 4 M-KOH (Haefner et al., 1977), extraction in chloroform and blowing dry under nitrogen gas. The D-glucosamine content of the aqueous phase was determined with an amino acid analyser.

32P-labelled *X. nematophilus* subsp. *nematophilus* var. *dutky* was obtained by growing the bacteria for 6 h in 10 litres of LB-broth containing 3-7 × 10⁶ Bq carrier-free [32P]phosphate. [32P]LPS and [32P]lipid A were extracted from the bacteria as described above.

LPS extracts were suspended in PBS by vortexing for ten 2 min periods and ultrasonicating for ten 1 min periods. The carbohydrate fractions were dissolved in PBS. Lipid A and total fatty acid fractions were dissolved in 0-5% (v/v) triethylamine in PBS.

**Toxicity assay for LPS and its fractions.** Toxicity of LPS and its fractions was based on the adhesion of selected bacterial species to the granular cells and plasmatocytes *in vitro* and on the elevation of larval haemocyte counts *in vivo*.

Unless otherwise stated, larvae were injected with 10 µl of solution containing either 2 µl LPS or 0-2 ng of lipid A or the total fatty acid extract. Control larvae received 10 µl PBS with and without 0-5% (v/v) triethylamine. Co-injections of LPS, lipid A and total fatty acid extracts with 10 mM-D-glucosamine (routinely detected in enterobacterial LPS (Wollenweber et al., 1982)) and N-acetyl-D-glucosamine [a common sugar associated with bacterial envelopes (Hammond et al., 1984)] and polymyxin B sulphate [which binds to lipid A (Teuber & Bader, 1976)] were done to ascertain the nature of the binding of these fractions to the haemocytes.

Insects were bled at designated times post-injection following the amputation of a thoracic proleg, and the haemocytes counted on a haemocytometer.

The influence of LPS on the adhesion of selected bacterial species to *G. mellonella* plasmatocytes and granular cells *in vitro* was assayed using a modification of the monolayer procedure of Ratcliffe et al. (1984). The prophenoloxidase-activating system, which may be part of the antibacterial response in insects (Leonard et al., 1985), exists in *G. mellonella* (Janssen, 1986). The antimelanizing agent 1-phenyl-Z-thioure (0-25 mg 1⁻¹) was added to the PBS used in these experiments to block phenoloxidase activity; the resulting mixture is referred to as
modified PBS. The monolayers were incubated in a nitrogen atmosphere so as to further decrease melanin formation and its possible influence on bacterial attachment to haemocytes (Dunn, 1986).

Slides with 100 µl modified PBS were inoculated with 100 µl haemolymph. The haemocytes were allowed to attach to the substrate for 5 min, then 100 µl modified PBS containing 6.2 µg X. nematophilus subsp. nematophilus var. dutky LPS was added to the haemocyte suspension and the mixture was incubated for 5 min at 27 °C on a platform shaker (100 r.p.m.). Test bacteria (B. subtilis, P. vulgaris, X. nematophilus subsp. nematophilus var. dutky) in 10 µl modified PBS were added to the slides and incubated for 5 min. The haemocyte : bacteria ratio was 1:10000. The slides were rinsed free of nonadhering bacteria and haemocytes (the latter consisting entirely of spherulocytes, prohaemocytes and oenocytoids) with modified PBS. The number of bacteria per plasmatocyte and per granular cell, and the percentage of these haemocytes with bacteria, was determined by examining 100 granular cells and plasmatocytes from each of 20 slides under the phase-contrast microscope. Phagocytosis was not measured.

[32P]LPS and [32P]lipid A binding to mixed haemocyte populations and isolated granular cells was initiated by adding 10 µl [32P]LPS (140000 d.p.m.) or [32P]lipid A (80000 d.p.m.) to microcentrifuge tubes containing 200 µl diluted haemolymph (1:3, v/v, with modified PBS) or 200 µl modified PBS containing granulocytes. Modified PBS was required to prevent melanization, which enhances LPS binding to the reaction vessels. The optimum incubation time for LPS and lipid A binding was 10 min at 20 °C. Thereafter, extensive haemocyte lysis occurred with a concomitant decline in LPS and lipid A binding. The haemocytes were centrifuge-washed twice (10000 g, 1 min, 15 °C) with modified PBS until no radioactivity was detected in the supernatant. Scintiverse II (1 ml) was added to each microcentrifuge tube followed by vortexing until the haemocyte pellet dispersed. The microcentrifuge tubes were placed into polyethylene scintillation counting vials and counted in a Beckman (LS 380) scintillation counter for 5 min per sample. Control samples consisted of (i) haemolymph in which the haemocytes had been lysed by ultrasonication and the solution converted to serum by centrifugation prior to the addition of the labelled test material, and (ii) intact haemocytes without the addition of labelled material. There was no significant difference between the scintillation counts of the two control groups, and so these groups were pooled and termed 'pooled controls'.

Haemocyte separation. The haemocytes from whole haemolymph were fractionated on a discontinuous Percoll (Sigma) gradient whose concentrations were 9, 17, 23, 29, 31 and 33% (v/v) in modified PBS (pH 6.5). The osmolality of each Percoll step was maintained with D-glucose at 430 mOsm, the osmolality of larval G. mellonella serum. Gradients were formed in 16 ml silanized round-bottomed polystyrene test tubes (Evergreen Scientific) using 1 ml volumes of the gradient solutions. Haemolymph (200 µl) was added to 800 µl of 9%, Percoll solution and layered evenly at the 9/17 Percoll interface. The test tubes were centrifuged in a swinging-bucket rotor (5000 g, 90 min, 5 °C). Haemocytes at the 29/31 interface consisted of 98% granular cells with a viability of at least 80%. The granular cells were centrifuge-washed (10000 g, 1 min, 5 °C) with modified PBS supplemented with D-glucose (12.7 g l-1). Granular cell viability averaged 87%. The granular cell fraction was used to determine the effects of D-glucose and its derivatives on [32P]LPS and [32P] lipid A binding.

SDS-PAGE. The Laemmli (1970) SDS-PAGE system was used in vertical slab gels to separate LPS molecules. The 5 cm stacking gel and 15 cm running gel of 4.5% and 12.5% (w/v) acrylamide, respectively, were subjected to 15 mA per gel. The gels were silver-stained (Tsai & Frasch, 1982).

Data analysis. Unless otherwise stated, data are expressed as the mean ± standard error of the mean. Percentages are expressed as the decoded mean of the 2 arcsin sqrt transformed data with 95% confidence limits. Changes in haemocyte counts were analysed using the Mann–Whitney U test (Sokal & Rohlf, 1969). The level of significance was P < 0.05.

RESULTS

The larvae injected with LPS from each of the three X. nematophilus subsp. nematophilus varieties had lower total haemocyte levels than did the PBS control larvae within 5 min of injection (P < 0.05) (Fig. 1). In LPS-injected larvae this was followed by a rapid increase in number of vacuolated haemocytes to maximum values at 20–35 min post-injection and a lytic decline thereafter. No nodules were detected. Within 1 h of injection of LPS, fat-body dissociation was observed based on the visual detection of individual fat-body cells and fat-body cell aggregates in the haemolymph of the larvae.

The LPS electrophoretic profiles of the breton and dutky varieties differed in detail from each other, (although both showed the ladder pattern of bands typical of smooth LPS), whereas those of the mexicanus and dutky varieties were identical to each other (not shown). The relative toxicity of the three LPS varieties was tested by injecting LPS into the larvae such that the levels of 3-deoxy-D-manno-octulosonic acid [determined by the thiobarbituric assay of Kharkhanis et
Fig. 1. Changes in total haemocyte counts of larval *G. mellonella* injected with LPS from *X. nematophilus* subsp. *nematophilus* var. *dutky* (▲), var. *breton* (▲) and var. *mexican* (○). Control larvae received PBS (●).

Fig. 2. Changes in total haemocyte counts (○) and endotoxin levels (●) in serum of larval *G. mellonella* following infection with live *X. nematophilus* subsp. *nematophilus* var. *dutky*.

*al. (1978)*] per 10 ml of LPS solution were the same. There was no significant difference in the rate of haemocyte increase between the different LPS varieties (rate = 1.4–1.2 × 10⁵ cells ml⁻¹ min⁻¹, *P* > 0.05).

Living or dead bacterial cells of each of the three varieties increased the haemocyte counts. The increase in the number of haemocytes was correlated with the presence of *Limulus* amoebocyte lysate coagulation factors (Fig. 2). Phenol/water extractions of infected larval serum produced factors which, in addition to coagulating the amoebocyte lysate, also elevated haemocyte counts at a rate not statistically different from that caused by the same amount of LPS from the homologous bacterial variety (*P* > 0.05) (Table 1). The serum extracts and envelope LPS for a given bacterial variety produced identical gel profiles on SDS-PAGE (not shown). Thus, the serum extracts are regarded as being LPS in nature.
Table 1. Effects of endotoxin cell envelope extracts from varieties of X. nematophilus subsp. nematophilus and of larval serum from G. mellonella infected by these bacteria on the increase in total haemocyte counts

<table>
<thead>
<tr>
<th>Bacterial variety</th>
<th>Extract source*</th>
<th>Rate of increase in haemocyte count (cells ml⁻¹ min⁻¹)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>dutky</td>
<td>Cell envelope</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Infected larval serum</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>breton</td>
<td>Cell envelope</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Infected larval serum</td>
<td>2.7 ± 0.1</td>
</tr>
<tr>
<td>mexican</td>
<td>Cell envelope</td>
<td>3.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Infected larval serum</td>
<td>2.8 ± 0.1</td>
</tr>
<tr>
<td>††</td>
<td>Uninfected larval serum</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

* Dose = 0.2 μg endotoxin per larva.
† Means ± standard error of the mean; n = 10 samples.
†† Control larval serum from uninfected larvae.

Table 2. Adhesion of selected bacterial species to larval G. mellonella haemocytes in vitro in the presence of X. nematophilus subsp. nematophilus var. dutky LPS and antimelanizing agents*

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Treatment†</th>
<th>Bacteria per haemocyte‡</th>
<th>Haemocytes with bacteria (%)§</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Granular cells</td>
<td>Plasmatocytes</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>PBS</td>
<td>5.0 ± 0.2</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>3.8 ± 0.4</td>
<td>3.8 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>LPS</td>
<td>2.1 ± 0.4</td>
<td>1.3 ± 0.0</td>
</tr>
<tr>
<td>Proteus vulgaris</td>
<td>PBS</td>
<td>5.5 ± 0.5</td>
<td>3.0 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>2.1 ± 0.4</td>
<td>1.3 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>LPS</td>
<td>1.2 ± 0.4</td>
<td>1.2 ± 0.0</td>
</tr>
<tr>
<td>X. nematophilus subsp. nematophilus var. dutky</td>
<td>PBS</td>
<td>1.1 ± 0.4</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>LPS</td>
<td>1.5 ± 0.4</td>
<td>2.8 ± 0.4</td>
</tr>
</tbody>
</table>

* Nitrogen atmosphere and PBS containing trace levels of phenylthiourea.
† PBS, Phosphate-buffered saline, pH 6.5, with trace amounts of phenylthiourea; LPS, lipopolysaccharide (6.2 μg per 100 μl PBS).
‡ Means ± standard error of the mean; n = 20 samples (100 haemocytes of each type examined per sample).
§ Means (with 95% confidence limits of 2 arcsin √p transformed data); n = 20 samples (100 haemocytes of each type examined per sample).

Incubating X. nematophilus subsp. nematophilus var. dutky with synthetic larval serum alone or synthetic larval serum with either washed intact or lysed haemocytes failed to elicit detectable amounts of endotoxin. However, in synthetic larval serum supplemented with larval serum, substantial endotoxin activity was detected within 4 h of inoculating the medium.

LPS influenced bacterial adhesion to G. mellonella plasmatocytes and granular cells in a manner that varied with the bacterial test species and the haemocyte type (Table 2). In general, LPS from the bacterial envelope of the dutky variety decreased bacterial adhesion to the granular cells and increased contact with the plasmatocytes (P < 0.05). LPS decreased adhesion of P. vulgaris to both types of haemocytes.

The toxicity of LPS from the three Xenorhabdus varieties was decreased by polymyxin B sulphate. LPS from the dutky variety was neutralized when the LPS:polymyxin B sulphate ratio was 51:1 (Fig. 3). The amount of antibiotic required for neutralization varied with the LPS.
Fig. 3. Effects on total haemocyte counts of injecting *X. nematophilus* subsp. *nematophilus* var. *dutky* LPS with increasing concentration of polymyxin B sulphate into larval *G. mellonella*. LPS (167 ng per larva) plus polymyxin B sulphate at 0 (●), 3.3 (▲), 16.7 (■) and 33.3 (■) ng per larva; polymyxin B sulphate alone at 33.3 ng per larva (▼); PBS (▲).

source and was stoichiometrically related to the LPS concentration. Increasing the concentration of polymyxin B sulphate for a given amount of LPS of the *dutky* variety increased the elevation of the haemocyte counts. Antibiotic alone elevated the haemocyte counts. Mixing LPS from the three *Xenorhabdus* varieties with excess polymyxin B sulphate followed by exhaustive dialysis against PBS to remove unbound antibiotic abrogated LPS toxicity.

Injecting LPS and the corresponding amounts of lipid A and total oligosaccharide from the LPS showed that only the carbohydrate fraction lowered haemocyte counts below the control levels (Fig. 4). Nodules were detected in the haemocoel in close proximity to the injection site in
Table 3. Binding of *X. nematophilus* subsp. *nematophilus* var. *dutky* [*32P*]LPS to larval *G. mellonella* haemocytes in vitro, in the presence of selected carbohydrates

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>Bound LPS (d.p.m.)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS†</td>
<td>460 ± 53</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>452 ± 49</td>
</tr>
<tr>
<td>D-Glucosamine</td>
<td>624 ± 66</td>
</tr>
<tr>
<td>N-Acetyl-D-glucosamine</td>
<td>365 ± 19</td>
</tr>
<tr>
<td>3-Deoxy-D-manno-octulosonic</td>
<td>405 ± 27</td>
</tr>
<tr>
<td>acid</td>
<td></td>
</tr>
<tr>
<td>D-Galactose</td>
<td>447 ± 34</td>
</tr>
<tr>
<td>D-Galactosamine</td>
<td>582 ± 21</td>
</tr>
<tr>
<td>N-Acetyl-D-galactosamine</td>
<td>357 ± 20</td>
</tr>
<tr>
<td>D-Mannose</td>
<td>468 ± 15</td>
</tr>
<tr>
<td>D-Fucose</td>
<td>846 ± 11</td>
</tr>
<tr>
<td>D-Trehalose</td>
<td>1751 ± 23</td>
</tr>
<tr>
<td>Pooled controls</td>
<td>27 ± 03</td>
</tr>
</tbody>
</table>

*Means ± standard error of the mean; n = 10 samples.
†Trace level of phenylthiourea present, to prevent melanization effects.

Table 4. Binding of [*32P*]LPS and [*32P*]lipid A of *X. nematophilus* subsp. *nematophilus* var. *dutky* to isolated *G. mellonella* granular cells in the presence of selected carbohydrates

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>Bound LPS (d.p.m.)*</th>
<th>Bound lipid A (d.p.m.)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS†</td>
<td>597 ± 26</td>
<td>275 ± 10</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>603 ± 31</td>
<td>283 ± 12</td>
</tr>
<tr>
<td>D-Glucosamine</td>
<td>851 ± 12</td>
<td>178 ± 05</td>
</tr>
<tr>
<td>N-Acetyl-D-glucosamine</td>
<td>254 ± 17</td>
<td>137 ± 12</td>
</tr>
</tbody>
</table>

*Means ± standard error of the mean; n = 10 samples.
†Trace level of phenylthiourea present, to prevent melanization.

larvae receiving the oligosaccharide. Lipid A induced a rapid rise in haemocyte numbers comparable to that caused by LPS.

Analysis for ninhydrin-positive compounds detected D-glucosamine in lipid A at approximately 22% (w/w). Co-injection of 10 mM D-glucosamine with lipid A prevented an increase in haemocyte counts. Polymyxin B sulphate neutralized lipid A activity when used in a 2:1 ratio. Both lipid A and LPS, at 0.5 mg per larva and 5 mg per larva, respectively, induced larval swelling, haemolymph coagulation and extensive fat-body destruction. These effects were blocked when the toxins were co-injected with 10 mM D-glucosamine.

To determine the stereospecific nature of the interaction between lipid A and the haemocytes, larvae were co-injected with lipid A plus 10 mM D-glucose, D-glucosamine, N-acetyl-D-glucosamine, D-galactose, D-galactosamine or N-acetyl-D-galactosamine. D-Glucosamine, N-acetyl-D-glucosamine, D-galactosamine and N-acetyl-D-galactosamine each neutralized the lipid A haemocytotoxin activity to a similar extent.

Binding of [*32P*]LPS from *X. nematophilus* subsp. *nematophilus* var. *dutky* to *G. mellonella* haemocytes was diminished by N-acetyl-D-galactosamine and N-acetyl-D-galactosamine and enhanced by D-glucosamine and D-galactosamine (Table 3) even though the latter two sugars prevented LPS-induced haemocyte damage in vivo. Similar results were obtained with isolated granular cells (Table 4). Both D-fucose and D-trehalose substantially increased LPS binding to mixed haemocytes (Table 3). Both D-glucosamine and N-acetyl-D-glucosamine diminished lipid A binding to the granular cells (Table 4).

The type of D-glucose derivative and its concentration influenced the binding of [*32P*]LPS and [*32P*]lipid A to mixed haemocytes. Both D-glucosamine and N-acetyl-D-glucosamine decreased
the binding of lipid A \( (P < 0.05) \) over the range of monosaccharide concentrations tested (Fig. 5). At 5 mM both sugars decreased LPS binding, but thereafter binding increased, with D-glucosamine having the greatest effect.

Injecting larvae with up to 100 \( \mu \)g phosphoethanolamine, ethanolamine and D-glucosaminyl-D-glucosamine, compounds associated with enterobacterial lipid A, did not elevate haemocyte counts or cause gross toxicity symptoms in the insects. Injecting larvae with the total fatty acid extracts from the lipid A of LPS from the \( dutky \) variety, and with lipid A providing a corresponding amount of fatty acids, elevated haemocyte counts (Fig. 6) at rates which were not statistically different \( (P > 0.05) \). The fatty acid effect could not be offset by either D-glucosamine or N-acetyl-D-glucosamine.

**DISCUSSION**

In the present study the haemocytotoxin in \( G. mellonella \) infected with \( X. nematophilus \) is LPS. The toxin in infected larval serum and in the phenol/water extract of the larval serum, like LPS from the bacterial cell envelope, induced \( Limulus \) amoebocyte coagulation. The serum extract and bacterial cell envelope LPS of homologous varieties induced similar rates of haemocyte damage when injected into the larvae, and the electrophoretic profiles of the two extracts were identical. The correlation of LPS release from varieties of \( X. nematophilus \) and haemocyte damage and re-emergence of \( X. nematophilus \) into the haemolymph (see Dunphy & Webster, 1984) establishes LPS as a component of the virulence mechanisms of \( X. nematophilus \).
LPS was released from both living and dead *X. nematophilus*, but only when the bacteria were exposed to humoral factors. This would explain the previously reported absence of toxins in *Xenorhabdus* spent media devoid of larval serum (Boemare et al., 1982; Dunphy & Webster, 1986). The reported absence of toxic activity in media supplemented with larval serum (Dunphy & Webster, 1986) may reflect the low levels of toxin production, the use of extensive gravity to remove bacteria from the medium and the use of relatively insensitive assay parameters, such as silk production and larval weight. Dunphy & Webster (1984) reported that living bacteria were not required in order for the toxin to be released. Although *G. mellonella* serum is believed to modify the envelope of *X. nematophilus* (Dunphy & Webster, 1984) it is not known how this relates to LPS release.

Short-term exposure of haemocyte monolayers to the dutky variety of LPS generally decreased adhesion of the test bacteria to the granular cells and enhanced bacterial contact with the plasmatocytes. Ratcliffe et al. (1984) reported that *Escherichia coli* O:55:B5 endotoxin enhanced the phagocytosis of *Bacillus cereus* by *G. mellonella* plasmatocytes and granular cells without activating the prophenoloxidase system. Although phenoloxidase activated by β-1,3-glucans and endotoxin may have a role to play in self/non-self recognition by arthropod haemocytes (Söderhall, 1981; Söderhall & Häll, 1984) endotoxin-triggered phenoloxidase does not occur in insects (Ashida et al., 1983; Leonard et al., 1985). However, to minimize any possible involvement of phenoloxidase in the present study, phenylthiourea and nitrogen gas were used to prevent phenoloxidase activation. Thus, larval *G. mellonella* haemocytes may initiate non-self responses by means of cell-surface lectins binding to selected carbohydrates associated with LPS. The ability of amino sugars to prevent haemocyte damage and to influence LPS and lipid A binding supports this contention. Enhancement of plasmatocyte activity by *X. nematophilus* LPS may represent either direct activation by LPS binding and/or indirect activation by granular cell discharge products, other than phenoloxidase, induced by LPS binding to the granular cells, perhaps in a cooperative manner as proposed by Ratcliffe et al. (1984). Haemocyte surface lectins and factors affecting plasmatocyte activity have been well documented for insects (Dunn, 1986).

*Shigella flexneri* LPS initiated nodulation in *G. mellonella* (Schwabel & Boush, 1971) and *Serratia marcescens* LPS and *E. coli* LPS triggered diffuse nodulation in *Schistocerca gregaria* and *Locusta migratoria* (Gunnarsson & Lackie, 1985). Nodulation may have occurred in *G. mellonella* injected with *X. nematophilus* LPS because the carbohydrate portion of the LPS induced nodulation. However, the survival of such nodules would be in doubt due to the haemocytotoxin activity of the LPS.

The observations that (1) the LPS gel profiles of the breton and dutky varieties differed, (2) injecting LPS samples containing the same concentration of 3-deoxy-α-manno-octulosonic acid produced comparable effects and (3) lipid A binding of polymyxin B sulphate neutralized LPS toxicity suggests that lipid A was the toxic moiety. This was confirmed by injecting lipid A into the larvae and neutralizing haemocyte damage, larval death and haemolymph coagulation with polymyxin B sulphate and selected amino sugars. Although LPS has been reported to influence haemocyte activity in arthropods (Kurstak et al., 1969; Newman & Feng, 1982; Goldberg & Greenberg, 1984; Gunnarsson & Lackie, 1985), induction of immunity in both insect (Chadwick & Vilk, 1969) and non-insect arthropods (Evans et al., 1969), and the induction of antibacterial proteins in mixed haemocyte and fat-body cultures (DeVerno et al., 1984), the active moiety of LPS has in most cases not been identified. Chadwick & Vilk (1969) reported that the oligosaccharide moieties of LPS induced immunity in *G. mellonella*, and Proctor & Textor (1985) discovered that the lipid A portion of *E. coli* LPS induced Limulus amoebocyte lysate coagulation. The present study is the first in insects to relate LPS toxicity to lipid A. It is known that most LPS toxic activity for mammals resides with the lipid A portion of the molecule (Galanos et al., 1977). Moreover, as with *Salmonella minnesota* (Wollenweber et al., 1982) and *Shigella flexneri* (Matsby-Baltzer et al., 1984), the fatty acids of *X. nematophilus* lipid A are responsible for endotoxin expression.

The haemocyte toxicity of LPS and lipid A was blocked with N-acetylated and non-acetylated amino sugars, suggesting that LPS, in part, binds to haemocytes by means of the glucosaminyl...
residue of lipid A. This was confirmed with the binding of $^{32}$P-LPS and $^{32}$P-lipid A to mixed haemocytes and purified granular cells.

The use of $^{32}$P-labelled LPS and lipid A, and of amino sugar inhibition of LPS and lipid A binding, produced unexpected results. Both D-glucosamine and N-acetyl-D-glucosamine decreased lipid A binding as the concentrations of the monosaccharides increased. However, the binding of $^{32}$P-LPS initially decreased and then increased with increasing monosaccharide concentration. The latter occurred even though in vivo toxic activity was suppressed. The binding profile of $^{32}$P-LPS to the granular cells in the presence of the inhibitory sugars suggests that two events occurred. At low amino sugar concentrations LPS binding would be impaired due, in part, to the inhibition of lipid A binding. The enhanced binding of LPS once the concentration of the amino sugar exceeded a critical threshold implies that the granular cells had been altered and become more adhesive for LPS. Localized granulocyte degradation that entraps foreign particles in a coagulum is known to occur in G. mellonella in response to non-self recognition (Ratcliffe & Rowley, 1979). Lackie (1979) proposed that non-self haemocyte responses would occur once a critical threshold of difference was exceeded. The present data support this. The decrease in lipid A binding may represent amino sugars blocking receptors and/or granular cells coagulum induced by the amino sugars either masking haemocyte receptors and/or creating an environment unfavourable to hydrophobic lipid A binding.

Both D-fucose and D-trehalose augmented the binding of $^{32}$P-LPS to the haemocytes. Fucose generally suppresses the adhesion of micro-organisms to larval haemocytes (Dunphy et al., 1986). The present result may reflect the use of simple molecules as opposed to the complex interaction of living micro-organisms with the sugars and the haemocytes. Analysis of bacterial adherence to eukaryotic cells may be complicated by bacterial surface changes (Smith, 1977) and by metabolism or modification of the monosaccharides used in sugar inhibition studies (McEachran & Irvin, 1985). The trehalose effect was unexpected because trehalose is a major haemolymph sugar in G. mellonella (Wyatt & Kalf, 1956) and so would be expected to be neutral in the binding of LPS to the haemocytes.

It is concluded that X. nematophilus releases LPS into larval G. mellonella haemolymph as a result of the undefined interaction between the bacterial envelope and larval humoral factors. LPS acts as a haemocytotoxin and may also be responsible for fat-body dissociation. Although the carbohydrate portion of the LPS binds to the haemocytes, it is the fatty acids of the lipid A moiety that express endotoxin activity. Lipid A binds to the haemocytes by means of D-glucosaminy1 residues to lectin-like molecules on the surfaces of the granulocytes.

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