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

By GARY B. DUNPHY*† AND JOHN M. WEBSTER

Department of Biological Sciences, Simon Fraser University, Burnaby, Vancouver,
Canada V5A 1S6

(Received 27 July 1987; revised 11 December 1987)

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.

† Present address: Department of Entomology, Macdonald College, McGill University, St. Anne de Bellevue, Quebec H9X 1C0, Canada.

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 entomophilic 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 trypticase 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-KCl, 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 × 10⁸ 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 ultrasonication 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-2-thiourea (0.25 mg l⁻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 μ l modified PBS were inoculated with 100 μ l haemolymph. The haemocytes were allowed to attach to the substrate for 5 min, then 100 μ l modified PBS containing 6.2 μ g *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 μ l 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 μ l [³²P]LPS (140000 d.p.m.) or [³²P]lipid A (8000 d.p.m.) to microcentrifuge tubes containing 200 μ l diluted haemolymph (1:3, v/v, with modified PBS) or 200 μ l 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 μ l) was added to 800 μ l 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 $\pm$ standard error of the mean. Percentages are expressed as the decoded mean of the 2 arcsin $\sqrt{p}$ 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 *bretton* 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 = $1.4\text{--}1.2 \times 10^5$ 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*

Bacterial variety	Extract source*	$10^{-5} \times$ Rate of increase in haemocyte count (cells $\text{ml}^{-1} \text{min}^{-1}$)†
<i>dutky</i>	Cell envelope	2.3 ± 0.1
	Infected larval serum	2.0 ± 0.2
<i>breton</i>	Cell envelope	2.5 ± 0.1
	Infected larval serum	2.7 ± 0.1
<i>mexican</i>	Cell envelope	3.1 ± 0.1
	Infected larval serum	2.8 ± 0.1
—‡	Uninfected larval serum	0.0 ± 0.0

* Dose = 0.2 μg endotoxin per larva.

† Means $\pm$ 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**

Bacterium	Treatment†	Bacteria per haemocyte‡		Haemocytes with bacteria (%§)	
		Granular cells	Plasmatocytes	Granular cells	Plasmatocytes
<i>Bacillus subtilis</i>	PBS	5.0 ± 0.2	1.3 ± 0.4	81 (2.346–2.165)	17 (0.976–0.795)
	LPS	3.8 ± 0.4	3.8 ± 0.5	40 (1.471–1.308)	52 (1.711–1.571)
<i>Proteus vulgaris</i>	PBS	5.5 ± 0.5	3.0 ± 0.4	94 (2.793–2.606)	70 (2.118–1.918)
	LPS	2.1 ± 0.4	1.3 ± 0.0	13 (0.850–0.676)	21 (1.047–0.850)
<i>X. nematophilus</i> subsp. <i>nematophilus</i> var. <i>dutky</i>	PBS	1.1 ± 0.4	1.4 ± 0.4	11 (0.795–0.574)	18 (0.952–0.823)
	LPS	1.5 ± 0.4	2.8 ± 0.4	8 (0.676–0.495)	51 (1.713–1.568)

* 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 $\pm$ standard error of the mean; $n = 20$ samples (100 haemocytes of each type examined per sample).

§ Means (with 95% confidence limits of 2 arcsin $\sqrt{p}$ 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 (▲).

Fig. 4. Changes in larval *G. mellonella* total haemocyte counts in response to *X. nematophilus* subsp. *nematophilus* var. *dutky* LPS (■), lipid A (●), total oligosaccharides from LPS (○), and PBS with (▲) and without (□) 0.5% triethylamine.

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* [32 P]LPS to larval *G. mellonella* haemocytes in vitro, in the presence of selected carbohydrates

Carbohydrate (10 mM)	Bound LPS (d.p.m.)*
PBS†	460 $\pm$ 53
D-Glucose	452 $\pm$ 49
D-Glucosamine	624 $\pm$ 66
N-Acetyl-D-glucosamine	365 $\pm$ 19
3-Deoxy-D-manno-octulosonic acid	405 $\pm$ 27
D-Galactose	447 $\pm$ 34
D-Galactosamine	582 $\pm$ 21
N-Acetyl-D-galactosamine	357 $\pm$ 20
D-Mannose	468 $\pm$ 15
D-Fucose	846 $\pm$ 11
D-Trehalose	1751 $\pm$ 23
Pooled controls	27 $\pm$ 03

* Means $\pm$ standard error of the mean; $n = 10$ samples.

† Trace level of phenylthiourea present, to prevent melanization effects.

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

Carbohydrate (10 mM)	Bound LPS (d.p.m.)*	Bound lipid A (d.p.m.)*
PBS†	597 $\pm$ 26	275 $\pm$ 10
D-Glucose	603 $\pm$ 31	283 $\pm$ 12
D-Glucosamine	851 $\pm$ 12	178 $\pm$ 05
N-Acetyl-D-glucosamine	254 $\pm$ 17	137 $\pm$ 12

* Means $\pm$ 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 [32 P]LPS from *X. nematophilus* subsp. *nematophilus* var. *dutky* to *G. mellonella* haemocytes was diminished by N-acetyl-D-galactosamine and N-acetyl-D-glucosamine 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 [32 P]LPS and [32 P]lipid A to mixed haemocytes. Both D-glucosamine and N-acetyl-D-glucosamine decreased

Fig. 5

Fig. 5. Binding of *X. nematophilus* subsp. *nematophilus* var. *dutky* [^{32}P]LPS to larval *G. mellonella* haemocytes in the presence of *N*-acetyl-D-glucosamine (▲) and D-glucosamine (△), and binding of [^{32}P]lipid A in the presence of *N*-acetyl-D-glucosamine (○) and D-glucosamine (●).

Fig. 6

Fig. 6. Changes in larval *G. mellonella* total haemocyte counts in response to injections of lipid A (▲), total fatty acid extracts of lipid A (○), 0.5% triethylamine in PBS (△) and PBS (●). Lipid A and its fatty acids were obtained from *X. nematophilus* subsp. *nematophilus* var. *dutky* LPS.

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 μg 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-D-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; Goldenberg & 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* (Mattsby-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 degranulation 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-glucosaminyl residues to lectin-like molecules on the surfaces of the granulocytes.

The authors thank Mr A. Syed (Simon Fraser University Insectary) for supplying the insects, Ms A. Erdman for advice with the use of [^{32}P]phosphate and Dr J. Chadwick (Queen's University, Kingston, Canada) for the *Proteus vulgaris* culture and consultation. This research was supported by grants from the Natural Sciences and Engineering Research Council of Canada and the Science Council of British Columbia.

REFERENCES

- AKHURST, R. J. (1980). Morphological and functional dimorphism in *Xenorhabdus* spp., bacteria symbiotically associated with the insect pathogenic nematodes *Neoaplectana* and *Heterorhabditis*. *Journal of General Microbiology* **121**, 303–309.
- ASHIDA, M., ISHIZAKI, Y. & IWAHANA, H. (1983). Activation of prophenoloxidase by bacterial cell walls or β -1,3-glucans in plasma of the silkworm *Bombyx mori*. *Biochemical and Biophysical Research Communications* **113**, 562–568.
- BERTANI, G. (1951). Studies on lysogenesis. I. The model of phage liberation by lysogenic *Escherichia coli*. *Journal of Bacteriology* **62**, 293–300.
- BOEMARE, N., LAUMOND, C. & LUCIANI, J. (1982). Mise en évidence d'une toxicogénèse provoquée par le nématode axénique entomophage *Neoaplectana car-pocapsae* Weiser chez l'insecte axénique *Galleria mellonella* L. *Comptes Rendus de l'Académie des Sciences* **295**, 543–546.
- CHADWICK, J. S. & VILK, E. (1969). Endotoxins from several bacterial species as immunizing agents against *Pseudomonas aeruginosa* in *Galleria mellonella*. *Journal of Invertebrate Pathology* **13**, 410–415.
- DEVERNO, P. J., CHADWICK, J. S., ASTON, W. P. & DUNPHY, G. B. (1984). The *in vitro* generation of an antibacterial activity from the fat body and hemolymph of non-immunized larvae of *Galleria mellonella*. *Developmental and Comparative Immunology* **8**, 537–546.
- DUNN, P. E. (1986). Biochemical aspects of insect immunology. *Annual Review of Entomology* **31**, 321–339.

- DUNPHY, G. B. & WEBSTER, J. M. (1984). Interaction of *Xenorhabdus nematophilus* subsp. *nematophilus* with the haemolymph of *Galleria mellonella*. *Journal of Insect Physiology* **30**, 883–889.
- DUNPHY, G. B. & WEBSTER, J. M. (1985). Influence of *Steinernema feltiae* (Filipjev) Wouts, Mracek, Gerdin and Bedding DD136 strain on the humoral and haemocytic responses of *Galleria mellonella* (L.) larvae to selected bacteria. *Parasitology* **91**, 369–380.
- DUNPHY, G. B. & WEBSTER, J. M. (1986). Influence of the Mexican strain of *Steinernema feltiae* and its associated bacterium *Xenorhabdus nematophilus* on *Galleria mellonella*. *Journal of Parasitology* **72**, 130–135.
- DUNPHY, G. B., MORTON, D. B., KROPINSKI, A. & CHADWICK, J. M. (1986). Pathogenicity of lipopolysaccharide mutants of *Pseudomonas aeruginosa* for larvae of *Galleria mellonella*: bacterial properties associated with virulence. *Journal of Invertebrate Pathology* **47**, 48–55.
- DUTKY, S. R., THOMPSON, J. V. & CANTWELL, G. E. (1962). A technique for mass rearing of the greater wax moth (Lepidoptera: Galleriidae). *Proceedings of the Entomological Society of Washington* **64**, 56–58.
- EVANS, E. E., WEINHEIMER, P. F., PAINTER, B., ACTON, R. T. & EVANS, M. L. (1969). Secondary and tertiary responses of the induced bactericidin from the West Indian spiny lobster, *Panulirus argus*. *Journal of Bacteriology* **98**, 943–946.
- GALANOS, C., LÜDERITZ, O., RIETSCH, E.-T. & WESTPHAL, O. (1977). Newer aspects of the chemistry and biology of bacterial lipopolysaccharides with special reference to their lipid A components. In *Biochemistry of Lipids II (International Review of Biochemistry vol. 14)*, pp. 239–335. Edited by T. W. Goodwin. Baltimore: University Park Press.
- GOLDENBERG, P. Z. & GREENBERG, A. H. (1984). Activation of lobster hemocytes for phagocytosis. *Journal of Invertebrate Pathology* **43**, 77–88.
- GUNNARSSON, S. G. S. & LACKIE, A. M. (1985). Hemocytic aggregation in *Schistocerca gregaria* and *Periplaneta americana* as a response to injected substances of microbial origin. *Journal of Invertebrate Pathology* **46**, 312–319.
- HAEFFNER, N., CHABY, R. & SZABO, L. (1977). Identification of 2-methyl-3-hydroxydecanoic and 2-methyl-3-hydroxytetradecanoic acids in the 'lipid X' fraction of *Bordetella pertussis* endotoxin. *European Journal of Biochemistry* **77**, 535–544.
- HAMMOND, S. M., LAMBERT, P. A. & RYCROFT, A. N. (1984). *The Bacterial Cell Surface*, pp. 1–28. Washington, DC: Kapitan Szabo Publishers.
- HOROHV, D. W. & DUNN, P. E. (1984). Role of hemocytotoxins in the pathogenicity of *Pseudomonas aeruginosa* in larvae of the tobacco hornworm, *Manduca sexta*. *Journal of Invertebrate Pathology* **43**, 297–298.
- JANSSEN, K. (1986). Investigation of the propheno-oxidase activating system in *Galleria mellonella*. *Developmental and Comparative Immunology* **10**, 624.
- KAMONEK, M. (1975). Effect of heat-killed cells of *Achromobacter nematophilus* Poinar et Thomas, and the fraction (endotoxin) isolated from them on *Galleria mellonella* L. caterpillars. *Bulletin de Académie Polonaise des Sciences* **23**, 277–281.
- KHARKHANIS, Y. D., ZELTNER, J. Y., JACKSON, J. J. & CARLO, D. J. (1978). A new and improved micro-assay to determine 2-keto-3-deoxyoctonate in lipopolysaccharide of Gram-negative bacteria. *Analytical Biochemistry* **85**, 595–601.
- KROPINSKI, A. M., KUZIO, J., ANGUS, B. L. & HANCOCK, R. E. W. (1982). Chemical and chromatographic analysis of lipopolysaccharide from an antibiotic-supersusceptible mutant of *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy* **21**, 310–319.
- KURSTAK, E., GORGING, I. & VEGA, C. (1969). Cellular defence in an arthropod in response to infection with a *Salmonella typhimurium* strain. *Antonie van Leeuwenhoek* **35**, 45–51.
- LACKIE, A. M. (1979). Cellular recognition of foreignness in two insect species, the American cockroach and the desert locust. *Immunology* **36**, 909–914.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- LEONARD, C., RATCLIFFE, N. A. & ROWLEY, A. F. (1985). The role of propheno-oxidase activation in non-self recognition and phagocytosis by insect blood cells. *Journal of Insect Physiology* **31**, 789–799.
- LYSENKO, O. (1976). Chitinase of *Serratia marcescens* and its toxicity to insects. *Journal of Invertebrate Pathology* **27**, 385–386.
- MCEACHRAN, D. W. & IRVIN, R. T. (1985). Adhesion of *Pseudomonas aeruginosa* to human buccal epithelial cells: evidence for two classes of receptors. *Canadian Journal of Microbiology* **31**, 563–569.
- MATTSBY-BALTZER, I., GERNSKI, P. & ALVING, C. R. (1984). Heterogeneity of lipid A: comparison of lipid A types from different Gram-negative bacteria. *Journal of Bacteriology* **159**, 900–904.
- NEWMAN, M. C. & FENG, S. Y. (1982). Susceptibility and resistance of the rock crab, *Cancer irroratus*, to natural and experimental bacterial infection. *Journal of Invertebrate Pathology* **40**, 75–88.
- POINAR, G. O., JR & HIMSWORTH, P. T. (1967). *Neoplectana* parasitism of larvae of the greater wax moth, *Galleria mellonella*. *Journal of Invertebrate Pathology* **9**, 241–246.
- PROCTOR, R. A. & TEXTOR, J. A. (1985). Activation and inhibition of *Limulus* amebocyte lysate coagulation by chemically defined substructures of lipid A. *Infection and Immunity* **49**, 286–290.
- RATCLIFFE, N. A. & ROWLEY, A. F. (1979). Role of hemocytes in defenses against biological agents. In *Insect Hemocytes, Development, Form, Functions and Techniques*, pp. 331–414. Edited by A. P. Gupta. New York: Cambridge University Press.
- RATCLIFFE, N. A. & WALTERS, J. B. (1983). Studies on the *in vivo* cellular reactions of insects: clearance of pathogenic and non-pathogenic bacteria in *Galleria mellonella* larvae. *Journal of Insect Physiology* **29**, 407–415.
- RATCLIFFE, N. A., LEONARD, C. & ROWLEY, A. F. (1984). Propheno-oxidase activation: nonself recognition and cell cooperation in insect immunity. *Science* **226**, 557–559.
- RUSSA, R., LÜDERITZ, O. & RIETSCH, E.-T. (1985). Structural analysis of lipid A from lipopolysaccharides of nodulating and non-nodulating *Rhizobium trifolii*. *Archives of Microbiology* **141**, 284–289.

- SCHWABEL, C. P. & BOUSH, G. M. (1971). Clearance of ^{51}Cr -labelled endotoxin from hemolymph of actively immunized *Galleria mellonella*. *Journal of Invertebrate Pathology* **18**, 85–88.
- SERYCZYNSKA, H. & KAMIONEK, M. (1977). Stimulation of the phagocyte cells of *Galleria mellonella* L. by microorganisms. *Bulletin de Académie Polonaise des Sciences* **25**, 173–176.
- SMITH, H. (1977). Microbial surfaces in relation to pathogenicity. *Bacteriological Reviews* **41**, 475–500.
- SÖDERHALL, K. (1981). Fungal cell wall beta-1,3-glucans inducing clotting and phenoloxidase attachment to foreign surfaces of crayfish hemocyte lysate. *Developmental and Comparative Immunology* **5**, 565–573.
- SÖDERHALL, K. & HÄLL, L. (1984). Lipopolysaccharide-induced activation of prophenoloxidase activating system in crayfish haemocyte lysate. *Biochimica et biophysica acta* **797**, 99–104.
- SOKAL, R. R. & ROHLF, F. J. (1969). *Biometry*, pp. 380–387. San Francisco: W. H. Freeman.
- TEUBER, M. & BADER, J. (1976). Action of polymyxin B on bacterial membranes. *Archives of Microbiology* **109**, 51–58.
- TSAI, C.-M. & FRASCH, C. E. (1982). A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Analytical Biochemistry* **119**, 115–119.
- WALTERS, J. B. & RATCLIFFE, N. A. (1983). Studies on the *in vivo* cellular reactions of insects: fate of pathogenic and non-pathogenic bacteria in *Galleria mellonella* nodules. *Journal of Insect Physiology* **29**, 417–424.
- WESTPHAL, O. & JANN, K. (1965). Bacterial lipopolysaccharides. Extraction with phenol-water and further applications of the procedure. *Methods in Carbohydrate Chemistry* **5**, 83–91.
- WOLLENWEBER, H.-W., BROADY, K. W., LÜDERITZ, O. & RIETSCHEL, E.-T. (1982). The chemical structure of lipid A. Demonstration of amide-linked 3-acyloxyacyl residues in *Salmonella minnesota* Re lipopolysaccharide. *European Journal of Biochemistry* **124**, 191–198.
- WYATT, G. R. & KALF, G. F. (1956). Trehalose in insects. *Federation Proceedings* **15**, 388.