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Detection of endotoxins with the Limulus test in burned and unburned mice infected with different species of gram-negative bacteria

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SUMMARY

The Limulus test detected endotoxins in the plasma of burned and unburned mice infected with different species of gram-negative bacteria. Individual strains of different species of gram-negative bacteria produced different amounts of endotoxin in the plasma of infected mice. Plasma from mice given lethal infections showed very high concentrations of endotoxin. Low concentrations of endotoxin in the plasma were tolerated by mice but high concentrations were invariably fatal. A polyvalent pseudomonas vaccine reduced endotoxin in the plasma of mice given lethal infections of *Pseudomonas aeruginosa*.

INTRODUCTION

In patients with burns, infection with gram-negative bacteria remains a major problem (Lowbury, 1972). Fortunately only a few of the bacteria are invasive, e.g. *Pseudomonas aeruginosa* and *Proteus mirabilis* (Jones & Lowbury, 1972). However, in patients with other diseases some of the non-invasive gram-negative bacteria found on burns are pathogenic, e.g. *Escherichia coli* (Cardis, Reinhold, Woodruff & Fine, 1972). In a burn, bacteria may be present for 1–3 weeks before the burned tissue is removed before grafting (Jones, 1975). During this time bacteria (invasive and non-invasive) are multiplying and could be releasing small amounts of endotoxin into the peripheral circulation, giving rise to symptoms ranging from a transient pyrexia (Jones, Jackson & Lowbury, 1966) to an acute endotoxaemia (Cuevas *et al.* 1974).

The Limulus test detects endotoxins in the plasma of patients infected with gram-negative bacteria (Levin & Bang, 1968) and recently it has been modified to detect very small amounts of endotoxin (Yin *et al.* 1972).

To find out the amount of endotoxin in plasma after infection, burned and unburned mice were given infections of varying severity with different species of gram-negative bacteria isolated from patients with burns, and their plasma was examined with the Limulus test for the presence of endotoxins.

To ascertain amounts of circulating endotoxin harmful to mice the Limulus test was used to measure the amount of endotoxin present in the plasma of groups of mice injected with lethal and sublethal doses of endotoxin.

An attempt was made to reduce the amount of endotoxin circulating in the plasma of burned mice infected with a lethal challenge dose of *Ps. aeruginosa* by injecting the burned mice before infection with a new polyvalent pseudomonas vaccine (Miler & Spilsbury, in preparation; Jones & Roe, in preparation).

MATERIALS AND METHODS

Extraction of lysate from Limulus polyphemus

King crabs (*Limulus polyphemus*) with 8–10 in. carapace obtained by Dr T. B. Brogan (The Welsh National School of Medicine) from Gulf Specimen Company, Inc., Panacea, Florida were kept in tanks containing sea-water by Mr P. Derne, at the Zoology Department, University of Birmingham.

Glassware. Special precautions were taken to ensure that glassware (syringes, universal containers with metal caps without rubber liners, tubes, pipettes, etc.) was clean and pyrogen-free. Brand new glassware was used throughout the experiments.

Cleaning. New glassware was immersed for 24 hr. in a glass-cleansing detergent (7X) supplied by the Central Public Health Laboratory, rinsed in hot distilled water, immersed in N/10 hydrochloric acid for 24 hr., rinsed in deionized water and dried in a hot air oven (160° C. for a minimum of 6 hr.). After cleaning, universal containers and syringes were siliconized in 'Repelcote'.

Removal of pyrogens. Pyrogens were removed by heating in a hot-air oven at 160° C. for a minimum of 6 hr. Before heating, syringes (barrel, cylinder and needles) were wrapped separately in aluminium foil and unplugged pasteur pipettes were placed in empty metal cannisters.

Preparation of lysate

The crab was inverted and tied to a wire basket and the cephalothorax was swabbed several times with methylated spirit. After the methylated spirit had dried a 100 ml. pyrogen-free siliconized syringe fitted with a 2·5 mm. bore needle was inserted into the cephalothorax to a depth of 1 cm. to the left-hand side of the median line (see Plate 1). Blood, consisting of amoebocytes (blood cells) and haemolymph (blue fluid), was drawn into the syringe which contained 50 ml. Tris-buffered 0·125% *N*-ethylmaleimide (NEM) pH 7·4. Once the syringe was filled, the blood was immediately transferred to 4 siliconized universal containers and centrifuged at 300 *g* for 6 min. The haemolymph was decanted from the deposited amoebocytes and without further washing the universal containers of amoebocytes were stored in the deep freeze (−21° C.).

Between 250 ml. and 300 ml. of blood/buffer solution was taken from each crab. Crabs treated this way could be bled again 2 months later.

When lysate for tests was required, the amoebocytes in the universal containers were thawed and resuspended in 2·0 ml. of pyrogen-free distilled water. The cell suspension was transferred to a capped, pyrogen-free, pyrex glass tube (15 × 10 cm.) and the amoebocytes were disrupted by freeze-thawing 10 times in liquid

nitrogen and a 37° C. water-bath. The cells were then centrifuged at 1200 ***g*** for 5 min. The slightly opalescent supernatant which contained the lysate was stored at 4° C. in a pyrogen-free container.

Standardization of lysate

To ensure that each preparation of lysate had similar activity, the lysate preparations were diluted with pyrogen-free distilled water so that they formed a gel when incubated for 2 hr. at 37° C. with 10^{-4} μg./ml. of lipopolysaccharide B *E. coli* O26:B6 (Difco Laboratories).

Limulus test

To 0·1 ml. of standardized Limulus lysate was added 0·1 ml. of material under test (plasma or solutions of endotoxin).

Endotoxins can be bound to plasma proteins and may be released by the 'pH shift' method described by Reinhold & Fine (1971). In these experiments all plasma was treated with the 'pH shift' method (*vide infra*). In the experiments 6 or 10 tenfold dilutions (0·1 ml.) of plasma in pyrogen-free saline were made; lysate (0·1 ml.) was added to all the tubes.

The test was performed in pyrogen-free capped 6 × 1 cm. glass tubes, and the period of incubation was 2 hr. in a 37° C. water-bath. Two control tubes were always included with each test: 0·1 ml. of lysate and 0·1 ml. of pyrogen-free saline (control for pyrogen-free conditions) and 0·1 ml. of lysate and 0·1 ml. of saline containing 10^{-4} μg./ml. of Difco *E. coli* O26:B6 endotoxin (control for stability of lysate).

After incubation, a solid gel or an increase in viscosity and turbidity was recorded as positive. The lysate/endotoxin control gave a solid gel while the lysate/saline control remained as a clear liquid.

Bacteria

Strains of *Ps. aeruginosa*, serotypes 1 and 6, *Pr. mirabilis* (10), *E. coli* (1), *Klebsiella aerogenes* (1), *Serratia marcescens* (1), *Pr. vulgaris* (1), *Pr. morganii* (1), *Providencia* sp. (1), *Acinetobacter anitratus* (1), and *Citrobacter* sp. (1), *Enterobacter cloacae* (1) and *Enterobacter* sp. (1) were isolated from patients with burns at the Burns Unit of the Birmingham Accident Hospital using the methods described by Davis, Lilly & Lowbury (1968). Strains of *Ps. aeruginosa* (13) were isolated from patients with burns at Safdarjang Burns Unit, Dehli, by Dr J. Gupta. The serological types of these strains were determined at Colindale by Mr T Pitt; strain 1 (serotype 1), 2 (5D Ps11), 3 (7), 4, 5, 11 and 13 (11), 6 and 9 (Ps11), 7 (3), 8 (4), 10 (10) and 12 (12). The LD100 of each of the bacteria used in the following experiments was determined by intraperitoneal (i.p.) injection (Jones, 1972).

Infection of mice

Mice (male, albino, Schofield) weighing 22–25 g. were infected i.p. by injecting 1·0 ml. of saline suspensions of gram-negative bacteria containing between

$7{\cdot}0 \times 10^7$ and $8{\cdot}4 \times 10^8$ bacteria/ml. Tables 1–4 show the bacteria and doses injected.

Mice were infected after burning by spreading 0·1 ml. of saline suspensions of various species of gram-negative bacteria over the surface of the burns 2 hr. after burning. Small standard burns (3 % of total body surface) were produced on depilated dorsal surfaces of anaesthetized mice by applying brass blocks heated in boiling water for 10 sec. (Roe & Jones, 1975).

Plasma from mice

Mice were anaesthetized by i.p. injection of 1·0 ml. of 1/20 dilution of Nembutal in saline. The ventral surface of the mice was swabbed with methylated spirit and allowed to dry. Cardiac puncture was used to draw about 1·5 ml. of blood into a 2·0 ml. plastic syringe containing 0·2 ml of pyrogen-free heparin (Evans Medical) in saline (100 units of heparin/ml. of pyrogen-free saline).

The blood was transferred to a pyrogen-free 10 × 1 cm. glass tube and the plasma was separated from the blood cells by centrifugation (2000 rev./min. for 10 min.). Plasma was stored in screw-top pyrogen-free containers at 4° C.

Endotoxins were freed from mouse plasma proteins by 'pH shift' method (Reinhold & Fine, 1971). To 1·0 ml. of mouse plasma was added 0·1 ml. of 25 % glacial acetic acid followed by 0·2 ml. of 50 % dipotassium hydrogen phosphate. The reaction was carried out in pyrogen-free conditions and the tubes were shaken after the addition of each reagent. The plasma was then used for the Limulus test.

As blood was taken from infected mice we were concerned that bacteria in the blood sample might cause the lysate to gel. Limulus lysate (0·1 ml.) was mixed with 0·1 ml. of 10-fold dilutions of suspensions of *Ps. aeruginosa*, P14, ranging between 10^7 and 10^1 bacteria. After incubation at 37° C. for 2 hr. only the tube containing 10^7 bacteria caused the lysate to gel. Plasma from infected mice was checked for the presence of bacteria by dropping 20 μl. of plasma on blood agar (4 % agar) and adding 20 μl. of plasma to nutrient broth. After overnight incubation at 37° C., bacteria were only found in plasma samples from moribund mice infected with *Ps. aeruginosa*.

EXPERIMENTAL PROCEDURES

Experiments using intraperitoneal infection

(1) To determine the amount of endotoxin in plasma of mice given a lethal infection of *Ps. aeruginosa* groups of 9 mice were infected with 1 LD100 of 13 different strains of *Ps. aeruginosa*. A pooled plasma sample was obtained from 3 mice from each of the 13 groups, 12 hr. and 24 hr. after infection. Endotoxin in the plasma was estimated by the Limulus test.

(2) To determine the amount of endotoxin in the blood of mice given lethal and sub-lethal proteus infections groups of 9 mice were infected with 10 different strains of *Pr. mirabilis*. Five of the groups of mice were infected with 1 LD50, strains 1–5; the other five groups of mice were infected with 1 LD70 (strain 6), 1 LD80 (strains 7 and 8), and 1 LD100 (strains 9 and 10). Pooled plasma from 3

mice was obtained from each of the 10 groups of mice 12 hr. and 24 hr. after infection.

(3) To determine the amount of endotoxin in plasma of mice 24 hr. after infection with 4 different doses (including 1 LD100) of bacteria from 10 different species of gram-negative bacteria, groups of 3 mice were infected with 0·2, 1·0, 2·0 and 4·0 $\times$ 10^9 of a strain of bacteria from each of the following bacterial species: *Ac. anitratus*, *Citrobacter* spp., *Ent. cloacae*, *Enterobacter* spp., *E. coli*, *K. aerogenes*, *Ser. marcescens*, *Ps. aeruginosa*, *Pr. vulgaris*, *Pr. morgani* and *Providencia* spp. Twenty-four hours after infection plasma was obtained from 3 mice from each group and the amount of endotoxin in each sample was estimated using the Limulus test.

Experiments using infected burned mice

To determine the amount of endotoxin in plasma of burned mice during 3 consecutive days after burning and infection with different gram-negative bacteria, burned mice were infected 2 hr. after burning with two doses (7·0 $\times$ 10^7 and 7·0 $\times$ 10^8) of 9 different species of gram-negative bacteria (see Table 4). Twelve mice were infected with each dose of each of the strains of bacteria. The endotoxin in pooled plasma from 3 mice was estimated by the Limulus test 1, 2 and 3 days after burning and infection. Plasma from uninfected burned control mice was examined in a similar manner.

Detection of endotoxin in plasma of mice injected with a pseudomonas vaccine

A vaccine, O1, prepared from a strain of *Ps. aeruginosa*, serotype 1, by Miler & Spilsbury (in preparation), was injected i.p. in 1·0 ml. amounts into groups of 18 mice at dilutions of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-5}. At $\frac{1}{2}$, 1, 2, 5, 8, 10 and 24 hr. after vaccination the amount of endotoxin in pooled plasma samples from 3 mice was determined by the Limulus test.

Experiments using vaccinated burned infected mice

To determine endotoxin levels in vaccinated and unvaccinated burned and infected mice, a group of 18 mice were infected on their burns with a lethal saline suspension (2·1 $\times$ 10^8) of a virulent strain of *Ps. aeruginosa*, P14, and another 18 mice were infected with a similar dose of an avirulent strain of *Ps. aeruginosa*, P1. A control group of 18 mice with burns spread with saline containing no bacteria were also included. To 9 mice from each of the 3 groups 1·0 ml. of a 10^{-2} dilution of polyvalent pseudomonas vaccine (Miler & Spilsbury, in preparation) was injected i.p. immediately after burning and before infection. Plasma was obtained from groups of 3 mice on days 1, 2 and 3 after burning and infection from each of the 6 groups of mice. Endotoxin in the plasma was estimated by the Limulus test.

Detection of endotoxin in plasma of mice injected i.p. with endotoxin

To determine the upper and lower limits of endotoxin circulating in the plasma of mice injected with lethal and sub-lethal amounts of endotoxin, groups of 24

Table 1. *Endotoxin in plasma from mice infected intraperitoneally with* 1LD100 *of* 13 *different strains of* Ps. aeruginosa

Time after infection when plasma examined with Limulus test (hr.)	Endotoxin (μg./ml.) in plasma from groups of 3 mice infected with 1 LD100 of 13 strains of *Ps. aeruginosa*													
	1	2	3	4	5	6	7	8	9	10	11	12	13	Saline
12	10	0·01	0·001	0·0	0·001	0·0	10	10	0·1	0·001	0·001	0·01	10	0
24	—*	—	—	0·01	0·01	0·0	—	—	—	0·001	—	—	10	0
Deaths (groups of 3 mice) at 24 hr.	3	3	3	2	2	2	3	3	3	2	3	3	2	0

* No sample obtainable.

LD100 of strains 1–6 was $2{\cdot}1 \times 10^8$, strains 7–12 was $4{\cdot}2 \times 10^8$ and strain 13 was $8{\cdot}4 \times 10^8$.

Table 2. *Endotoxin in plasma from mice infected intraperitoneally with* Proteus mirabilis

Time after infection when plasma examined by Limulus test (hr.)	Endotoxin (μg./ml.) in plasma from groups of 3 mice infected with 10 strains of *Pr. mirabilis* at the following doses										
	LD50					LD70	LD80		LD100		
	1*	2	3	4	5	6	7	8	9	10	Saline
8	10	1·0	0·01	0	0	0·1	10	0	10	10	0
24	10	1·0†	10	10	0	0·1	–‡	10	10	10	0

* Strain number of *Proteus mirabilis*.
† Sample taken from one mouse only.
‡ All mice died.

mice were injected i.p. with 1·0 ml. of saline containing 0·2, 0·5, 1·0 and 2·0 mg./mouse of endotoxin (Difco, *E. coli* O26:B6). At ½, 2, 4, 6, 8, 12, 18 and 24 hr. after injection of endotoxin plasma was obtained from 3 mice (where enough mice had survived the challenge) from each of the four challenge groups. The amount of endotoxin in the plasma was measured by the Limulus test. Mortality was also recorded.

RESULTS

Endotoxin in plasma of mice infected i.p. with gram-negative bacteria

Infection with lethal doses of Ps. aeruginosa

Table 1 shows the amounts of endotoxin in pooled plasma (groups of 3 mice) in mice injected i.p. with 1 LD100 of 13 different strains of *Ps. aeruginosa* together with amounts of endotoxin in plasma of mice who survived for 24 hr. after infection. Twelve hours after pseudomonas infection the endotoxin concentrations in the plasma were found to differ by as much as 10,000-fold (10 – 0·001 μg./ml. of plasma) between some groups of infected mice.

Of the few mice which survived the challenge for 24 hr. the endotoxin concentration was either the same as that found at 12 hr. or slightly higher. No endotoxins were found in plasma of mice infected with *Ps. aeruginosa* strains 4 and 6, or in the plasma of control uninfected mice.

Pseudomonas strains 1–6 were twice as virulent as strains 7–12, and 4 times as virulent as strain 13 as judged by the number of bacteria required to kill mice by i.p. infection. Table 1 shows that there was no correlation between the amount of endotoxin in plasma of mice 12 hr. after infection and the number of bacteria injected i.p., e.g. mice infected with the relatively avirulent strain 13 had as much endotoxin in their plasma as mice infected with 4 times less bacteria of strain 1.

Infection of mice with lethal and sub-lethal doses of Pr. mirabilis

Table 2 shows the amount of endotoxin in the plasma of groups of 3 mice 8 hr. and 24 hr. after infection (i.p.) with similar numbers (1×10^9) of 10 different strains of *Pr. mirabilis*.

Table 3. *Endotoxin in plasma from mice infected intraperitoneally with a range of doses of different species of gram-negative bacteria*

Dose of bacteria injected (i.p.) ($\times 10^8$)	Endotoxin (μg./ml.) in plasma from groups of 3 mice 24 hr. after i.p. infection with gram-negative bacteria										
	Acinetobacter anitratus	*Citrobacter* species	*Enterobacter* species	*Enterobacter cloacae*	*Escherichia coli*	*Klebsiella aerogenes*	*Serratia marcescens*	*Pseudomonas aeruginosa*	*Proteus vulgaris*	*Proteus morgani*	*Providencia* species
0·2	0	0	0	0	0	0	0·001	0	0	0	0
1·0	0	0	1	0	0·01	**0·001**	**0·01**	0	0	0	0·001
2.0	0	**10**	**1**	0	**0·01**	0·01	—	**0·001**	0	**0·1**	**0·1**
4·0	**0**	0	0·1	**0·1**	—	—	—	—	**0·001**	—	—

Figures in bold type indicate LD100 of infecting bacteria.

Table 4. *Endotoxin in plasma from mice with burns infected with different species of gram-negative bacteria*

Bacteria inoculated on to the burn		Endotoxin (μg./ml.) in plasma from groups of 3 mice on following days after burning and infection		
Type	Dose	1	2	3
Ps. aeruginosa (P1)	$7{\cdot}0 \times 10^7$	0·0	0·0	0·0
Ps. aeruginosa (P14)	$7{\cdot}0 \times 10^7$	0·0	1·0	0·0
Ser. marcescens	$7{\cdot}0 \times 10^7$	0·0	0·0	0·0
Providencia spp.	$7{\cdot}0 \times 10^7$	0·0	0·0	0·0
Citrobacter spp.	$7{\cdot}0 \times 10^7$	0·0	0·0	0·0
K. aerogenes	$7{\cdot}0 \times 10^7$	0·0	0·0	0·0
E. coli	$7{\cdot}0 \times 10^7$	0·0	0·0	0·0
Pr. mirabilis	$7{\cdot}0 \times 10^7$	0·0	0·0	0·0
Pr. morgani	$7{\cdot}0 \times 10^7$	0·0	0·0	0·0
Ps. aeruginosa (P1)	$7{\cdot}0 \times 10^8$	0·0	0·1	0·0
Ps. aeruginosa (P14)*	$7{\cdot}0 \times 10^8$	10·0	1·0	10·0
Ser. marcescens	$7{\cdot}0 \times 10^8$	0·0	10·0	0·0
Providencia spp	$7{\cdot}0 \times 10^8$	0·01	0·0	0·0
Citrobacter spp.	$7{\cdot}0 \times 10^8$	0·01	0·0	0·0
K. aerogenes	$7{\cdot}0 \times 10^8$	0·01	0·1	0·0
Pr. mirabilis	$7{\cdot}0 \times 10^8$	0·0	0·0	0·0
Pr. morgani	$7{\cdot}0 \times 10^8$	0·01	0·001	0·0
Uninfected burned control mice		0·0	0·0	0·0

* *Ps. aeruginosa* (P14) 7×10^8 = 1 LD100.

Mice infected with 1 LD50 (strains 1–5) showed a wide range of endotoxin in their plasma 8 hr. after infection: at 24 hr. after infection the amounts of endotoxin in these mice were the same as or higher than they had been at 8 hr. A similar wide range of endotoxin (0–10 μg./ml. of plasma) was found in the plasma of mice infected with LD70 and LD80 of *Pr. mirabilis*. Mice infected with 1 LD100 of *Pr. mirabilis* (strains 9 and 10) showed large amounts (10 μg./ml. of plasma) of endotoxin 8 hr. and 24 hr. after infection. No endotoxin was found in the plasma of uninfected mice.

Infection with different species of gram-negative bacteria

Mice were infected with doses (including LD100) of 10 different species of gram-negative bacteria (Table 3): 24 hr. later only mice infected with *Ac. anitratus* showed no detectable endotoxin in their plasma at any of the 4 infective doses. Very few (4/23) of the plasma samples from mice infected with gram-negative bacteria at infective doses of less than 1 LD100 contained endotoxin. At the lowest infective dose ($0{\cdot}2 \times 10^8$) only mice infected with *Ser. marcescens* showed endotoxin in their plasma. All mice which looked ill (hunched appearance, immobile, diarrhoea) at the time of sampling were found to have endotoxin in their plasma.

Table 5. *Endotoxin in plasma from mice injected intraperitoneally with pseudomonas vaccine* O1*

Dilution of vaccine O1 injected	Endotoxin (μg./ml.) in plasma from groups of 3 mice at times after vaccination (hr.)						
	½	1	2	5	8	10	24
10^{-1}	0·01	0·01	0·01	0	0	0	0
10^{-2}	0·01	0·01	0·01	0	0	0	0
10^{-3}	0·001	0	0	0	0	0	0
10^{-5}	0·0	0	0	0	0	0	0

* Vaccine O1 prepared at Wellcome Research Laboratories.

Endotoxin in plasma of mice with infected burns

Burned mice were infected with 8 different species of gram-negative bacteria isolated from burned patients (Table 4).

No endotoxin was found in plasma from control mice with uninfected burns, on any of the 3 days after burning.

At the lower of the two infective doses used in the experiment (7×10^7) only mice infected 2 days with *Ps. aeruginosa* P14 showed endotoxin (upper half of Table 4) in their plasma. Mice infected with another strain of *Ps. aeruginosa* P1, less virulent than *Ps. aeruginosa* P14 (Roe & Jones, 1975), showed no endotoxin in their plasma. At the higher infective dose (7×10^8) all groups of infected mice, except for those with burns infected with *Pr. mirabilis*, showed endotoxin in one or more of the samples taken on the 3 days after infection. Mice infected with *Ps. aeruginosa* P14 were the only mice with endotoxin in their plasma on all 3 days after infection, and were also the only group of mice with endotoxin in their plasma on the 3rd day after burning and infection.

All burned mice infected with 7×10^8 bacteria were observed for 7 days after burning even though the Limulus test was only performed on samples taken on the first 3 days after burning and infection. Mice infected with *Ps. aeruginosa* P14 died 4 days after infection: high concentrations of endotoxin were found on all 3 days before death. None of the mice infected with other bacteria died: these mice showed low concentrations (0·001 – 0·1 μg./ml. of plasma) of endotoxin, except those infected with *Ser. marcescens* which showed a severe localized infection of the burn, and were ill for 2 or 3 days after infection: these mice showed a high concentration of endotoxin in their plasma on the 2nd day after burning, although none of the mice died.

Endotoxin in plasma from mice injected with pseudomonas vaccine

The Limulus test showed that low concentrations (0·01 – 0·001 μg./ml. of plasma) of endotoxin were present in the plasma of mice for up to 2 hr. after injection of 1 ml. of pseudomonas O1 vaccine diluted 10^{-1} or 10^{-2}. Mice injected with 10^{-3} of pseudomonas vaccine showed a small amount (0·001 μg./ml.) of endotoxin in their plasma for a short period (½ hr.). A dose of 10^{-5} of vaccine was undetectable (Table 5).

Table 6. *Endotoxin in plasma from vaccinated* and unvaccinated burned infected mice*

Bacteria inoculated on burn		Polyvalent pseudomonas vaccine	Endotoxin (μg./ml.) of plasma from groups of 3 mice on following days after burning, vaccination and infection		
Type	Dose ($\times 10^8$)		1	2	3
Ps. aeruginosa (P1)	2·1	—	0·1	0·1	0·0
Ps. aeruginosa (P14)	2·1	—	10·0	1·0	10·0
Uninfected burned controls	0·0	—	0·0	0·0	0·0
Ps. aeruginosa (P1)	2·1	+	0·1	0·1	0·0
Ps. aeruginosa (P14)	2·1	+	0·001	0·001	0·1
Uninfected burned controls	0·0	+	0·0	0·0	0·0

* Vaccine – 1·0 ml. of polyvalent pseudomonas vaccine injected intraperitoneally immediately after burning and before infection.

Endotoxin in plasma from burned vaccinated mice

Plasma from burned mice taken 1, 2 or 3 days after the burns were infected with a lethal dose of *Ps. aeruginosa* P14 showed large amounts of endotoxin (10·0, 1·0, 10 μg./ml. of plasma); by the following day (4th day after burning) all the mice had died. A similar group of mice vaccinated (polyvalent pseudomonas vaccine) after burning showed much less endotoxin in their plasma (Table 6): none of these mice died within 4 days of burning.

Vaccination did not alter the low concentration of endotoxin in the plasma of mice infected with an avirulent strain of *Ps. aeruginosa* (P1).

Endotoxin in plasma of mice injected i.p. with endotoxin

The amounts of endotoxin circulating in the plasma of mice after i.p. injection of 0·2, 0·5, 1·0 or 2·0 mg./mouse of endotoxin (*E. coli*) are shown in Fig. 1.

Maximum amounts of endotoxin (10^2 and 10^6 μg.) were found in the plasma 2 and 4 hr. after injection of the two lower doses (0·2 and 0·5 mg./mouse) of endotoxin. Once the peak of endotoxin in the plasma had been reached it gradually decreased and became undetectable 12 hr. after injection.

Four hours after mice were injected with 1·0 mg./mouse of endotoxin the maximum of circulating endotoxin was reached. This high concentration (10^6 μg./ml. of plasma) was maintained in the plasma for 4 hr. then it began to decrease and became undetectable 18–24 hr. after injection. Two in three of mice injected with 1·0 mg./mouse died 18 hr. after the endotoxin had been injected.

Mice injected with the lethal dose of endotoxin (2·0 mg./mouse) showed lower concentrations of endotoxin in their plasma during the first 8 hr. after injection than mice injected with 1·0 mg./mouse of endotoxin. At 8 hr. the maximum of 10^6 μg. of endotoxin/ml. of plasma was reached. Twelve hours after injection 1/3 mice had died and they had all died by 18 hr.

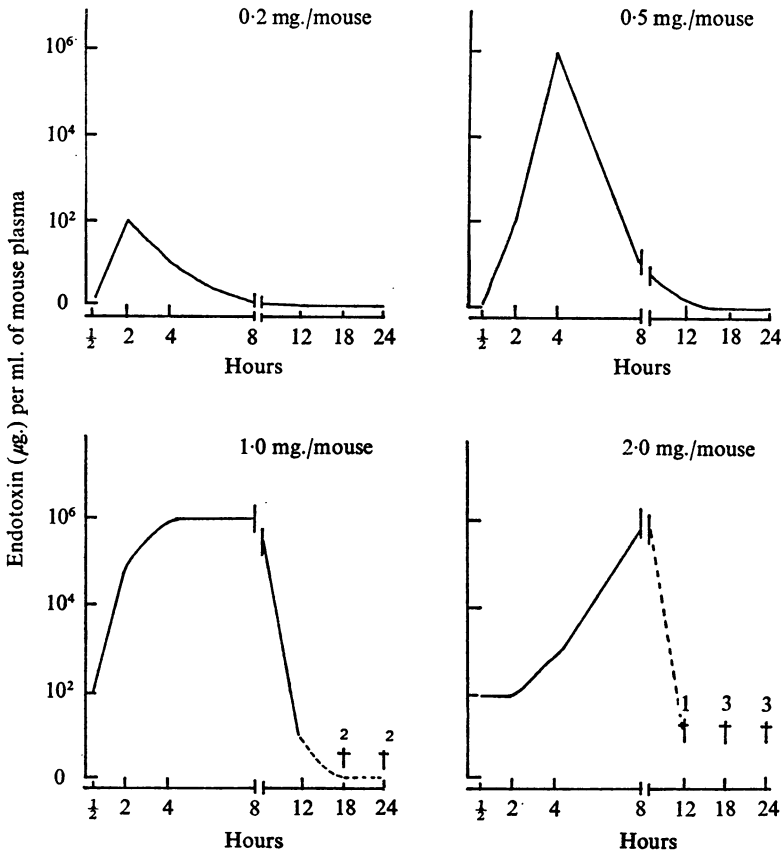

Fig. 1. Endotoxin (μg./ml.) in plasma from mice injected intraperitoneally with *E. coli* endotoxin. †, Number of mice which died per group of three.

DISCUSSION

Most bacteria isolated from patients with burns produced detectable endotoxins in the plasma of burned and unburned mice provided the mice were infected with sufficiently large doses (especially lethal) of bacteria, and provided that the Limulus test was performed within 24 hr. of infection. The invasive bacterium (*Ps. aeruginosa*, P14) produced larger amounts of endotoxin sooner than the non-invasive bacteria (e.g. *Providencia* spp. *or Citrobacter* spp.) and this was most marked in mice given lethal infective doses. It was thought to be of significance that the highest concentrations of endotoxin recorded in plasma of the infected mice were found in samples taken just before death.

Two strains of *Pr. mirabilis*, one inoculated on a burn and the other injected i.p., and a strain of *Ac. anitratus* injected i.p. failed to produce endotoxins in mouse plasma. It is possible that these strains do not produce endotoxins *in vivo* because our experiments showed that the liberation of endotoxin was found to vary from strain to strain in mice infected (i.p.) with 10 strains of *Pr. mirabilis* and also with 13 different strains of *Ps. aeruginosa*. The range of endotoxin con-

centrations found in mice infected with similar numbers of bacteria differed by as much as 10,000-fold between one strain and another.

In interpreting the results of the experiments with infected mice it was a great advantage to have estimated the presence of endotoxin in serial samples of plasma because in several groups of the mice endotoxin was only found in 1/4 samples taken after infection. A rising titre of endotoxin or consecutive high titres of endotoxin in a series of daily samples could be taken as an index of a potentially fatal infection (e.g. mice infected with *Ps. aeruginosa*, P14) whereas a single sample which contained a high titre of endotoxin could either be due to a transient appearance of endotoxin (e.g. *Ser. marcescens*) or could be the start of endotoxaemia (e.g. *Ps. aeruginosa*, P14).

The presence of an endotoxin in plasma need not be regarded as an adverse sign for the mouse because mice were found to tolerate low (0·1 – 0·001 μg./ml. of plasma) but detectable amounts of endotoxin in their blood for short periods of time without any apparent ill effects. High concentrations of endotoxin (10^6 μg./ml.) remaining in the plasma for more than 4 hr. were invariably fatal and were found only in mice given artificially large doses of endotoxin. Fatal infections produced lower amounts of endotoxin in the blood (1·0 – 10 μg./ml.) than were found after injection of lethal doses of endotoxin but they persisted for 3 days after infection, whereas sub-lethal injections of endotoxin had disappeared by 12 hr.

The Limulus test detected endotoxins in plasma of mice infected with a wide range of gram-negative bacteria and is therefore a non-specific test. But while the test gave a good chance of detecting endotoxins resulting from gram-negative infections of burns, there was no certainty that the bacteria on the burn were the only bacteria contributing to the endotoxin circulating in the plasma.

A practical point which emerged from these experiments was that bacteria themselves will gel Limulus lysate. Thus one must be sure that samples examined for endotoxins are bacteria free. This can be simply done by filtration through washed Millipore GS filters or by culturing part of the blood sample or the reaction tube after gelation. It is possible that false positives recorded by some users of the Limulus test were due to the presence of bacteria growing in tubes during incubation.

The varying amounts of endotoxin found in the plasma of infected mice are difficult to interpret. The experiments suggest that a threshold of endotoxin tolerance exists in mice; amounts of endotoxin remaining above threshold were fatal while those below the threshold were tolerated. It was found that if one could lower the amount of endotoxin in plasma, e.g. by vaccination, then mice challenged with a lethal infective dose would survive. The beneficial effect of vaccination is obviously not solely attributable to its ability to lower the endotoxin in plasma of infected mice; it will also prime other immune systems, e.g. enhance the phagocytic ability of polymorphs (Jones & Dyster, 1973).

In the near future it is proposed to vaccinate burned patients with the polyvalent pseudomonas vaccine and the experiments suggest that monitoring patients with the Limulus test might be useful since one would not expect high concentrations of endotoxin to be found in the plasma of the vaccinated patients.

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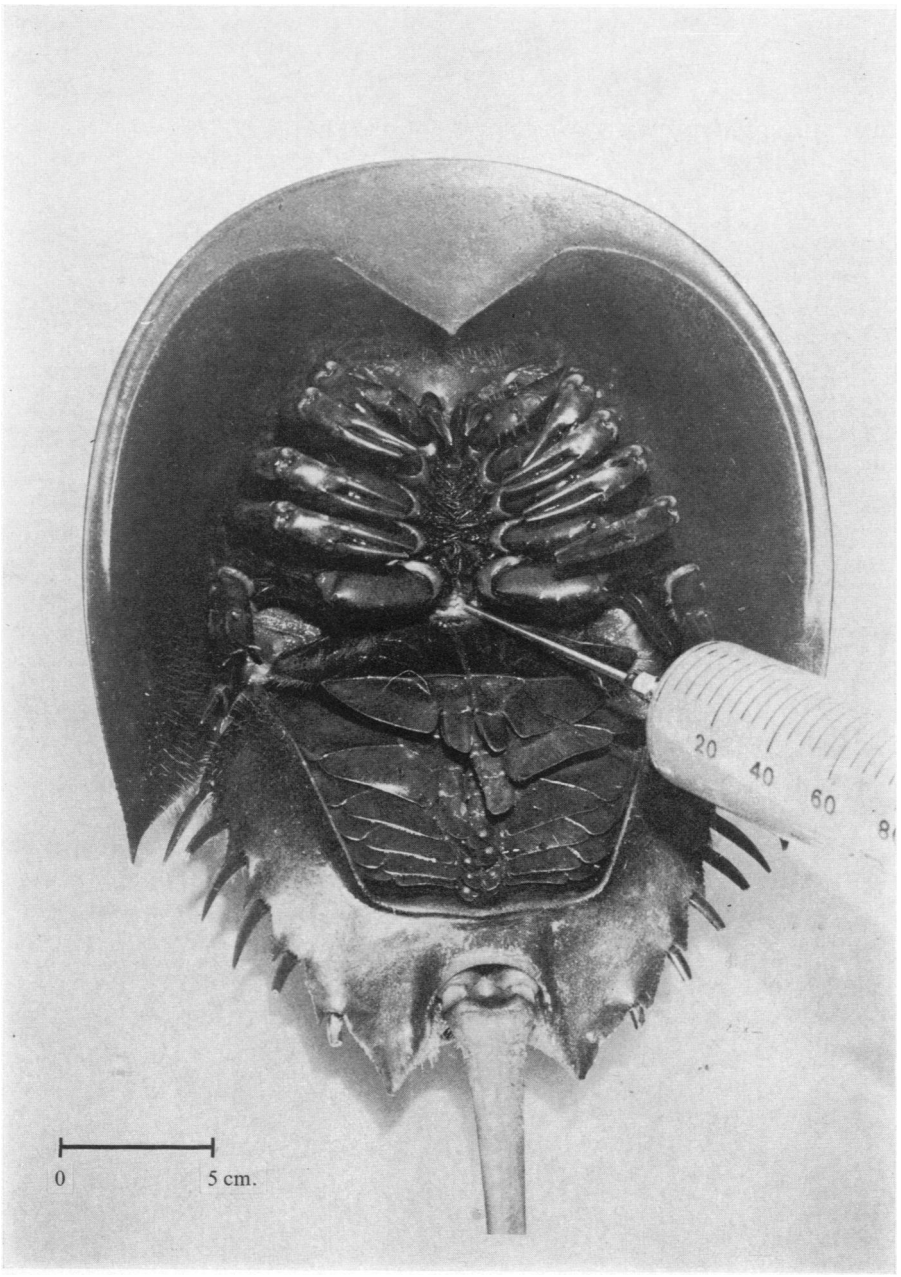
EXPLANATION OF PLATE

Bleeding of *Limulus polyphemus*.


20
40
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5 cm.