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Measurement of endotoxins with the limulus test in burned patients

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SUMMARY

High titres of endotoxin as measured by the Limulus test were usually found in burned patients who had raised body temperatures, and were colonized with gram-negative bacteria; also some infected patients showed raised endotoxin without a raised temperature.

Patients vaccinated with an antipseudomonas vaccine rarely showed endotoxin in their plasma but occasional plasma samples from vaccinated patients had a high titre of endotoxin which appeared unrelated to infection or to a raised temperature in the patient.

INTRODUCTION

Infection with gram-negative bacteria is a constant hazard of patients with burns (Lowbury, 1976) and tests which aid early recognition of this group of bacteria help to expedite diagnosis and treatment. The Limulus test is a rapid, sensitive *in vitro* method for detecting or estimating titres of endotoxins from gram-negative bacteria in blood plasma (Caridis *et al.* 1972; Yin *et al.* 1972; Jones, Roe & Dyster, 1975).

In this study the Limulus test was used to measure endotoxin in plasma samples taken weekly from burned patients, starting on the day of admission to hospital. The bacteria present or the burns were monitored by routine methods (Davis, Lilly & Lowbury, 1966). Some burned patients were vaccinated with a new polyvalent pseudomonas vaccine (Jones *et al.* 1976; Miler *et al.* 1977) which increases resistance to the most invasive of the gram-negative bacteria, *Pseudomonas aeruginosa* (Jones, Roe & Gupta, 1978).

MATERIALS AND METHODS

Patients

Adults, 18–65 years, with burns more than 15% of their body surfaces admitted to the Burns Unit, Birmingham Accident Hospital, were selected for study. Eighteen patients were part of a controlled clinical trial of a pseudomonas vaccine (Jones *et al.* 1976; Miler *et al.* 1977). Nine patients were vaccinated subcutaneously once a week for 3 consecutive weeks with pseudomonas vaccine (PEV–01, Wellcome

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Research Laboratories, Kent, England) and 9 other patients served as unvaccinated controls. All 18 patients were given similar antibacterial chemotherapy (silver sulphadiazine (Lowbury, 1976)). Of a further 18 patients studied 9 were treated prophylactically with cream containing silver phosphate-chlorhexidine and 9 with silver sulphadiazine (Babb *et al.* 1977).

Blood samples

An attempt was made to take blood under pyrogen-free conditions. The site of venepuncture was carefully wiped with alcohol (74 o.p.) containing 0·5% chlorhexidine gluconate. Five ml of blood was carefully drawn into a pyrogen-free plastic syringe (Sabre, Gillette) and put into a 10 ml pyrogen-free heparinized plastic tube (Searle). Blood was centrifuged (4000 rev./min for 10 min) and plasma removed to pyrogen-free glass tubes and treated by the 'pH shift method' (Rheinhold & Fine 1971) to liberate endotoxin from the plasma proteins.

Limulus lysate assay

Tenfold dilutions of plasma (0·1 ml) ranging from 10^{-1} to 10^{-10} treated by the 'pH shift method' of Rheinhold & Fine (1971) to liberate endotoxin from the plasma proteins, were made in pyrogen-free distilled water. Standardized Limulus lysate 0·1 ml (Jones, Roe & Dyster, 1975) gelling with 1·0 pg/ml of Difco *Eschericia coli* O26:B6 endotoxin, was mixed with each 0·1 ml plasma dilution in pyrogen-free stoppered glass tubes and incubated at 37 °C for 30 min. A solid gel, which could not be poured from the tube when inverted, was read as positive. Control tubes containing the lysate alone, the lysate with the distilled water for dilution and the lysate with 1·0 pg of Difco *E. coli* O26:B6 endotoxin (positive control) were included in each batch of plasma tested.

Bacteriology

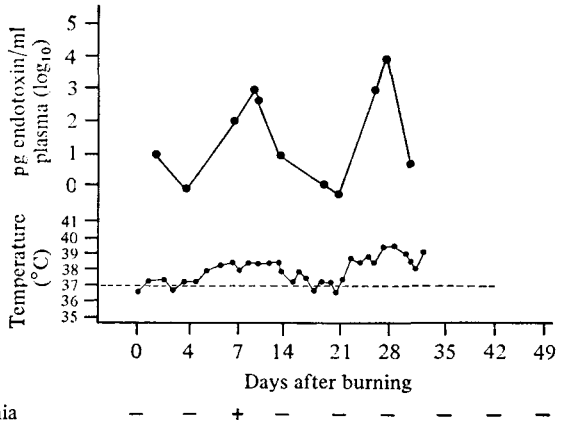
Swabs from the burns were taken at each change of dressing (every 3–4 days) and cultured on 4% blood agar. Growth from the agar was identified according to the methods of Davis *et al.* (1966).

RESULTS

The endotoxin in the plasma samples (45) from healthy volunteers ranged from 0–10 pg/ml blood. Figs. 1–4 show endotoxin titres in plasma, body temperature and bacteriology of burns of four burned patients.

In the patient shown in Fig. 1 (30% of body surface full-skin-thickness burns) there was a close association between fluctuations in body temperature and endotoxin levels in plasma. The first rise in endotoxin occurred when the burns became colonized with gram-negative bacteria and a second peak occurred a few weeks later when the heaviest growth of *Ps. aeruginosa* was found on the burns (days 14–28). The patient was colonized with *Staphylococcus aureus* during the whole of her stay in hospital and once *S. aureus* was grown on blood culture (day 9, Fig. 1).

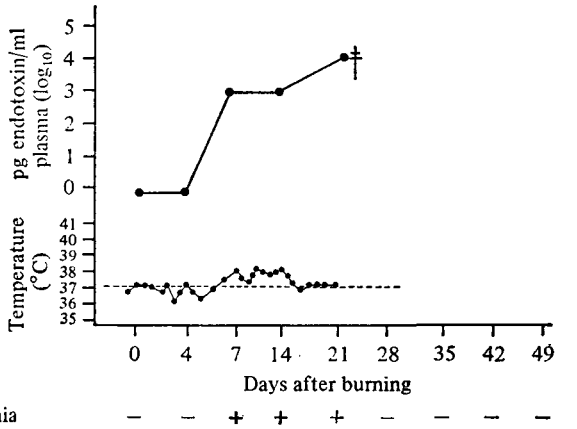
In a burned patient who died with an *E. coli* septicaemia the endotoxin in the



Septicaemia – – + – – – – – –

Bacteria on burn
Ps. aeruginosa – – – + + + + + –
Other g-ve – – + + + + + + +
Staph. aureus – + + + + + + + +

Fig. 1. EAB 30%. Age 31 years.



Septicaemia – – + + + – – – –

Bacteria on burn
Ps. aeruginosa – – – – +
Other g-ve – – + + +
Staph. aureus – – + + +

Fig. 2. EAB 40%. Age 79 years.

plasma increased steadily (Fig. 2). The body temperature was only slightly raised during the week before death even though the endotoxin rose steadily. *E. coli*, *Klebsiella aerogenes*, *Proteus mirabilis* and *S. aureus* were isolated from the burns in the 2 weeks before death. *Ps. aeruginosa* was isolated only on the day before he died.

No endotoxin was found in any plasma sample from patient shown in Fig. 3. The patient was heavily colonized with *S. aureus* for 12 days after burning, and showed a raised body temperature at this time but otherwise body temperature remained around the average. No gram-negative bacteria were isolated from this patient.

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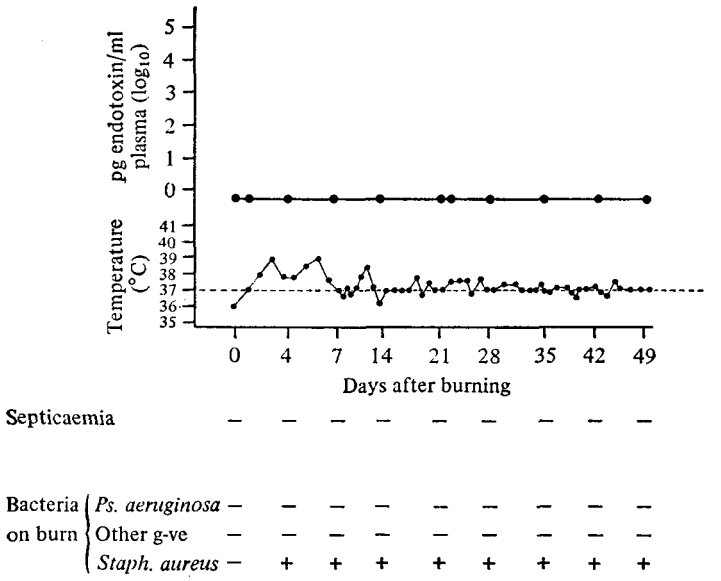

Fig. 3. EAB 20%. Age 49 years.

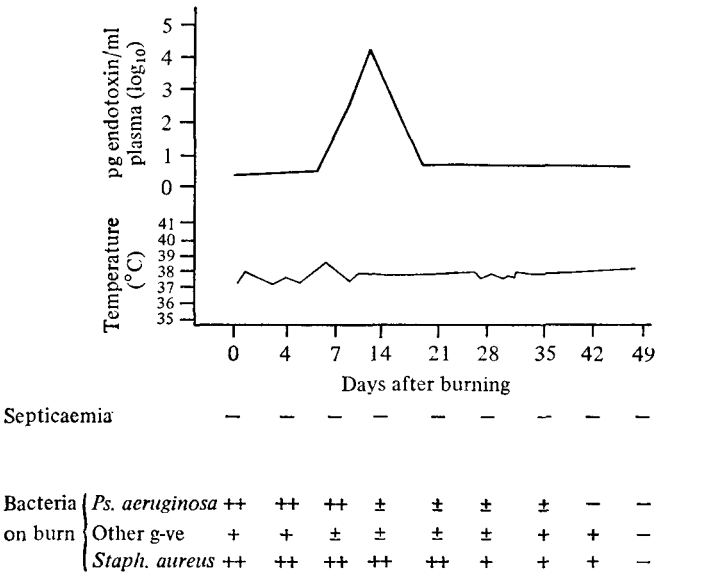

Fig. 4. EAB 20%. Age 26 years.

Fig. 4 shows an example of a patient who had been vaccinated with an anti-pseudomonas vaccine. When gram-negative bacteria including *Ps. aeruginosa* were present on the burn only one sample of plasma showed endotoxin.

Table 1 shows the range of endotoxin levels in serial samples from vaccinated and unvaccinated patients with burns. The majority of samples from patients in each group contained small amounts of endotoxin (10 pg/ml or less), patients receiving the antipseudomonas vaccine had more samples with low endotoxin than patients in the other two groups ($\chi^2 = 5{\cdot}9$ $0{\cdot}02 > P > 0{\cdot}01$).

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Table 1. *Endotoxin in plasma of vaccinated* and unvaccinated burned patients*

Patients	No. of patients	No. of samples	No. (%) of plasma samples With endotoxin titres (pg/ml) 0–10	10^2–10^3	10^4–10^5
Vaccinated	9	45	41 (91)	2 (4·5)	2 (4·5)
Unvaccinated	22	116	83 (72)	21 (18)	12 (10)

* Antipseudomonas vaccine.

Table 2. *Endotoxin in the plasma of patients with infected burns*

Bacteria on burn when plasma obtained	No. of plasma samples	No. (percentage) of plasma samples with endotoxin titres (pg/ml) 0–10	10^2–10^3	10^4–10^5
Ps. aeruginosa, *S. aureus*, *Coliforms*	38	24 (63)	5 (13)	9 (24)
Coliforms, *S. aureus*	14	7 (50)	6 (43)	1 (7)
S. aureus	94	86 (91)	8 (9)	0 (0)
None (admission samples*)	25	22 (88)	2 (8)	1 (4)

* Sample taken within 12 h of burning (2–3 h after admission to hospital).

Plasna samples with high endotoxin were often found in patients with mixed infections, *Ps. aeruginosa*, *S. aureus* and miscellaneous coliforms (Table 2). In contrast, patients colonized with only *S. aureus* at the time the sample was taken had the lowest endotoxin titres in their plasma. Patients colonized with gram-negative bacteria had proportionally more of their plasma samples with high endotoxin than patients colonized with *S. aureus*. Plasma taken from patients on admission to hospital when burns are usually sterile (Jones, 1974) also showed low values of endotoxin,

DISCUSSION

The highest titres of endotoxin (10^3–10^5 pg/ml) were found in the plasma of burned patients whose burns were infected with gram-negative bacteria, especially *Ps. aeruginosa*, and this was often associated with a raised body temperature. Patients were not found to have high amounts of endotoxin in their plasma on admission, probably because most burns were not colonized with bacteria at this time (Jones, 1974). Endotoxin in plasma of burned patients above 10 pg/ml (the upper limit for our healthy volunteers) was only found in patients with bacteria colonizing their burns. Burned patients with endotoxin in plasma of 10^3 pg/ml or more, especially with a rising titre of endotoxin in serial samples of plasma, were usually infected with *Ps. aeruginosa*, *Pr. mirabilis*, *K. aerogenes*, or other coliforms and showed raised body temperatures. The highest endotoxin titres in plasma (10^5 pg/ml) were mainly found in patients infected with *Ps. aeruginosa* for 1 week or longer. This high titre of endotoxin is extremely dangerous for the patient, and

the patient who died (Fig. 2) with a plasma endotoxin titre of 10^5 pg/ml showed a fall in body temperature before death which is symptomatic of endotoxin shock.

The usefulness of the Limulus test in burns is that it detects a harmful titre of endotoxin in plasma thereby differentiating between infection and colonization with gram-negative bacteria in a patient with a raised body temperature. Its disadvantage is that it is a non-specific test and does not confirm the species of bacterium producing the endotoxin. The Limulus test itself presented no practical problems once techniques for operating under 'pyrogen-free' conditions in the laboratory were achieved; the most difficult part of the test was establishing 'pyrogen free' conditions at the time the blood was taken. The test could be read 1 h after taking the blood and was useful for prognosis.

Vaccination with an antipseudomonas vaccine reduced the number of plasma samples from burned patients which contained above 10 pg/ml of endotoxin. This is an encouraging sign because circulating endotoxin is known to increase a patient's susceptibility to infection (Clark, 1978) and depletes C3 complement pathway (Roitt, 1975), and suggests that early vaccination is likely to strengthen a patient's resistance to infection not only against *Ps. aeruginosa* but other species of bacteria as well, by preventing high titres of endotoxin damaging protective immune mechanisms.

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