Biochemical Pharmacology, Vol. 34, No. 22, pp. 3995–3998, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

DOSE-DEPENDENT REDUCTION OF LIPOPOLYSACCHARIDE PYROGENICITY BY POLYMYXIN B

STEPHEN J. C. WARNER,*† DUNCAN MITCHELL,‡ NERINA SAVAGE† and ELIZABETH MCCLAIN‡

Departments of †Medical Biochemistry and ‡Physiology, University of the Witwatersrand Medical School, Parktown, 2193 Johannesburg, Republic of South Africa

(*Received* 16 *November* 1984; *accepted* 18 *June* 1985)

Abstract—Lipopolysaccharides (LPS) from Gram-negative bacteria are potent pyrogens in mammals. Polymyxin B (PB), a cationic polypeptide antibiotic, binds lipid A, the active moiety of LPS, with high affinity and abrogates several biological responses to LPS. We studied the effect of PB on pyrogenicity of purified LPS from *E. coli* 0111:B4 in rabbits. PB reduced the pyrogenic response to LPS in a dose-dependent manner at mass ratios (PB:LPS) from 5:1 to 100:1. Previous reports have suggested that PB is effective only at much higher doses. In our hands, PB itself is pyrogenic, unless previously gamma-irradiated. Our results confirm *in vivo* the anti-endotoxic action of PB.

Lipopolysaccharides (LPS), the endotoxins derived from Gram-negative bacterial cell walls, are potent pyrogens. They exert their effect by inducing the release of an endogenous pyrogen (interleukin 1) from mononuclear phagocytes [1]. The cationic polypeptide antibiotic, polymyxin B (PB), abolished LPS-induced release of endogenous pyrogen from rabbit monocytes *in vivo* [2]. The pyrogenicity of LPS in rabbits is mediated by the lipid A moiety of the molecule [3], and high-affinity binding of PB to lipid A has been demonstrated [4]. We have therefore investigated the effect of PB on the pyrogenicity of purified LPS in rabbits. Previous studies of this effect have suffered from two shortcomings: the LPS preparations were not highly purified, and large amounts of PB relative to LPS were used, obscuring any dose–response characteristics. We report here a dose-dependent reduction of LPS pyrogenicity by concentrations of PB from 5 to 100 times those of LPS.

MATERIALS AND METHODS

Reagents

Phenol-extracted LPS from *E. coli* 0111:B4 was obtained from Sigma Chemical Company (St. Louis, MO). PB (8137 units mg^{-1}) was from Calibiochem (La Jolla, CA). Pronase and ribonuclease were supplied by Boehringer (Mannheim, F.R.G.). All other reagents were of analytical grade.

LPS purification

Crude LPS was dissolved at 10 mg ml^{-1} in phosphate-buffered saline (0.15 M NaCl, 0.01 M phosphate buffer, pH 7.0) and dialysed against several changes of the same solution, until the diffusate showed no absorption above background at a wavelength of 256 nm (A_{256}). The dialysed LPS was incubated with 25 μg ml^{-1} ribonuclease and dialysed against phosphate-buffered saline until the A_{256} of the diffusate again was zero. Pronase (25 μg ml^{-1}) was then added, and dialysis continued at room temperature overnight [5]. The LPS was then dialysed repeatedly against distilled water at 4°, lyophilized and stored at 4° until used.

The protein content of the purified LPS was determined according to Lowry *et al.* [6]. Colitose was measured in crude and purified LPS by the thiobarbituric acid assay [7]. Finally, the purified LPS was analysed on 12.5% polyacrylamide gels containing 0.1% sodium dodecyl sulphate (SDS) and 4 M urea. Bands were detected by silver staining [8].

Animals

Conscious New Zealand white rabbits of both sexes, weighing between 2.4 and 3.3 kg, were restrained in conventional wooden stocks.

Temperature measurements

Temperatures were recorded by means of copper–constantan thermocouples inserted about 100 mm into the rectum. All thermocouples were calibrated by water-immersion against a certified mercury thermometer. Temperatures were monitored at 10-min intervals on a data logger. Temperatures were recorded for at least 60 min prior to injections. Animals exhibiting variations of basal temperature greater than 0.4° and febrile animals were excluded from the study.

Solutions and injections

Purified LPS was dissolved (1 mg ml^{-1}) in sterile, pyrogen-free saline. Dilutions to achieve the desired final concentration were made just before injection. PB was dissolved in sterile, pyrogen-free saline at 10 mg ml^{-1}. In some experiments PB and LPS were

* To whom all correspondence should be addressed.

incubated together for 30 min at room temperature prior to dilution and injection. Volumes of 1 to 2 ml were injected into an ear marginal vein. Experiments were performed at least 7 days apart to avoid the possible development of tolerance to LPS pyrogenicity.

One batch of PB was subjected to γ-irradiation in a ^{60}cobalt source (Atomic Energy of Canada Ltd.) A 1 mg ml^{-1} solution of PB was exposed to 1500 rads of irradiation.

Quantitation of fever responses

Temperature responses were calculated as the rise in rectal temperature (δT) above that prevailing at the time of injection. The thermal response index (TRI) was calculated for each treatment [9]; this index is the time integral of δT for a period of 3 hr following injection and is reported as °hr. All data are given as the mean ± standard error of the mean (S.E.M.) of responses in a minimum of three rabbits for each treatment.

RESULTS

LPS purification

Commercially supplied LPS contained significant amounts of protein, RNA and dialysable contaminants. LPS recovery during purification was monitored by assaying for colitose, a constituent of the *O*-polysaccharide of *E. coli* 0111:B4 LPS. More than 90% of the colitose present in the crude LPs was recovered in only 47% of the original mass. We found phenol-extracted LPS from both Sigma and Difco to be impure.

SDS–polyacrylamide gel electrophoresis of purified LPS revealed a typical pattern of bands (Fig. 1). Incubation of the purified LPS with pronase or ribonuclease prior to electro-phoresis did not alter the pattern, so that none of the bands was likely to have been protein or RNA. The purified LPS also was free of protein as determined by the procedure of Lowry *et al.* [6] and was shown to be free of RNA by the lack of the characteristic absorbance peak at 250–260 nm (Fig. 2).

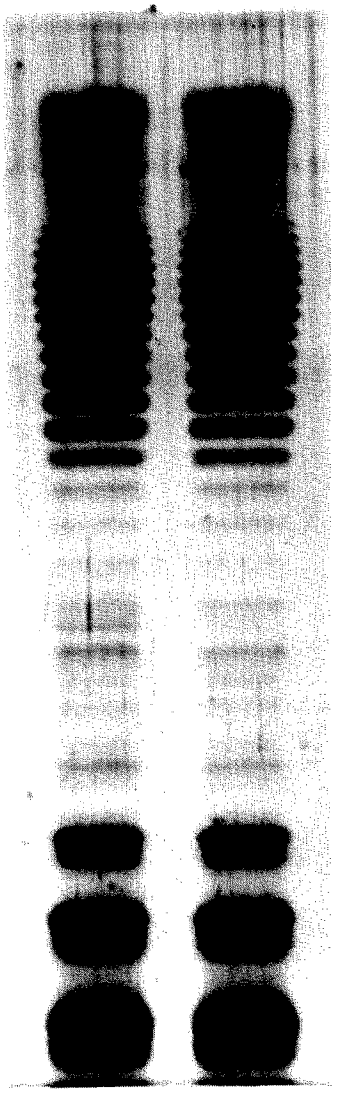

Fig. 1. SDS–polyacrylamide gel electrophoresis of purified lipopolysaccharide from *E. coli* 0111:B4. LPS (10 μg) was analysed on a 12.5% polyacrylamide gel and silver stained. The results are in excellent agreement with those of Tsai and Frasch [8].

Pyrogenicity of purified LPS

The dose–response data for purified LPS are shown in Fig. 3. The doses of 10 ng–1 μg correspond to about 3.4–340 ng kg^{-1} mass.

Dose-dependent reduction of LPS pyrogenicity by PB

Figure 4 shows the thermal response index following injection of 1 μg LPS, either alone or with 5, 10, or 100 μg polymyxin B. The PB reduced the fever below that evident following the LPS alone, but only at the 5:1 PB:LPS ratio was the reduction statistically significant ($P < 0.01$, unpaired *t*-test). As Fig. 4 shows, the failure of higher doses of PB to produce significant reduction in LPS pyrogenicity resulted, at least partly, from an inherent pyrogenicity of the PB itself.

Since there have been no previous reports indicating that PB might be pyrogenic in its own right, we assume that the PB had become contaminated with an extraneous pyrogen. Considering that PB is derived from *Bacillus polymyxa*, and the fact that the PB we used was not intended for human drug use, this is not unlikely.

In an attempt to eliminate the pyrogen, we γ-irradiated a batch of PB and the consequences of pre-incubating purified LPS with this PB are shown in Figs 5 and 6. There was no significant fever following injection of 10 μg of irradiated PB alone, and pre-incubation at 100:1 PB:LPS ratio reduced the TRI resulting from 100 ng LPS to 38% of the LPS value.

DISCUSSION

We have shown that PB-treated LPS is significantly less pyrogenic than LPS alone. The reduction of

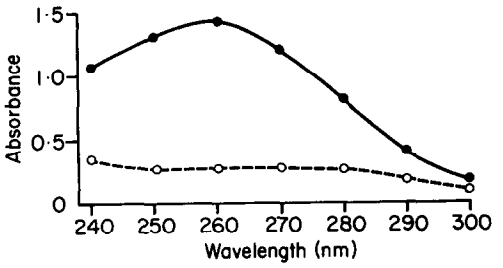

Fig. 2. Ultraviolet absorbance spectra of crude (●) and purified (○) LPS. Spectra were determined in a Beckman 35 spectrophotometer on 0.4 mg ml^{-1} LPS in distilled water.

pyrogenic response was dose-related over a range of PB:LPS ratios from 5:1 to 100:1. Other biological activities of LPS which are inhibited by PB include lethality in mice [10] and in chick embryos [11, 12], erythrocyte haemagglutination [13], inhibition of macrophage migration [14], disseminated intravascular coagulation [15], the Shwartzman reaction [16] and mitogenicity in spleen cells [17]. PB was effective in neutralizing the toxicity of *E. coli* 0111:B4 LPS in chick embryos at a PB:LPS ratio of 115:1 [11], while the corresponding ratio in mice was 300:1 [10]. Erythrocyte haemolysis mediated by *E. coli* 0111:B4 LPS was inhibited at a PB:LPS ratio 4:1 [13].

An early attempt to reduce the pyrogenic effect of

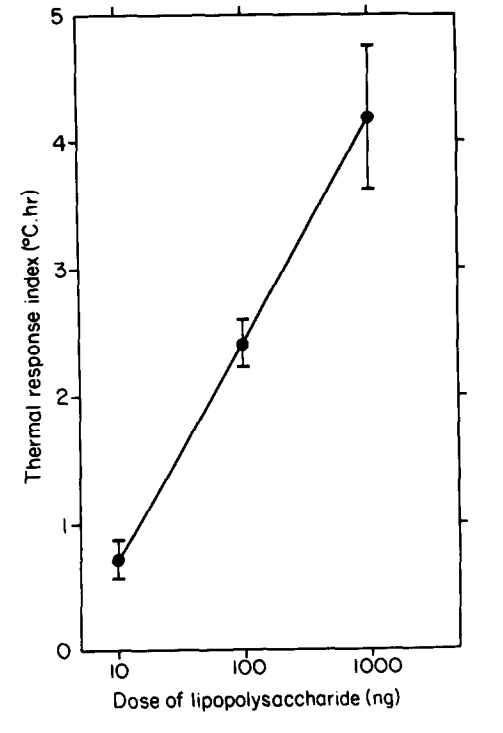

Fig. 3. Three-hour thermal response index (mean ± S.E.M.) in four rabbits following i.v. injection of purified LPS. The doses are much lower than those of crude LPS required to produce the same TRI [3].

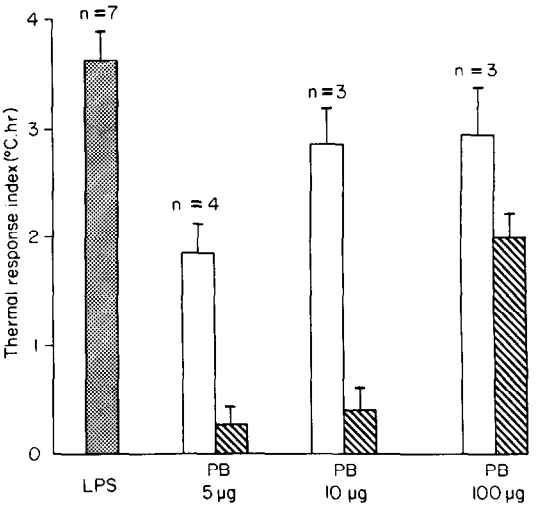

Fig. 4. Thermal response index following i.v. injection of 1 µg purified LPS (approx. 340 ng kg^{-1}), either alone or after 30 min pre-incubation with 5, 10 or 100 µg polymyxin B (unshaded bars). Shaded bars represent the TRI following i.v. injection of the same dose of PB alone.

LPS with PB was unsuccessful [18], but Van Miert and Van Duin [19] reported a reduction of LPS-induced fever by PB pre-treatment. Incubation of 0.4 µg LPS with PB at PB:LPS ratios of 2.5×10^3:1 to 10^4:1 produced TRIs ranging from 46 to 34% of control values. The LPS used in these early studies [19], was obtained from commercial sources, and was extracted from an unspecified serotype of *E. coli*. In contrast we have shown, using highly purified LPS from *E. coli* 0111:B4, that the TRI was reduced to 50% of control values by pre-treatment with only five times the mass of PB.

Galanos *et al.* [20] have pointed out the intrinsic variability in the physico-chemical properties and biological activities of LPS extracted by various methods from different bacterial sources. Commonly used extraction methods yield preparations contaminated with other biologically active molecules, such as protein and ribonucleic acids. It is not surprising then, that variability in the pyrogenic potency

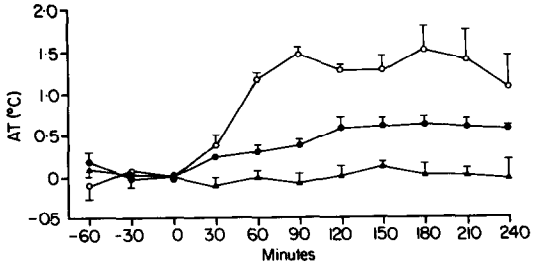

Fig. 5. Rise in rectal temperature (mean ± S.E.M.) following i.v. injection at time zero of 0.1 µg purified LPS, (○, N = 3), 0.1 µg LPS pre-incubated with 10 µg irradiated PB (●, N = 3), or 10 µg irradiated PB alone (Δ, N = 4).

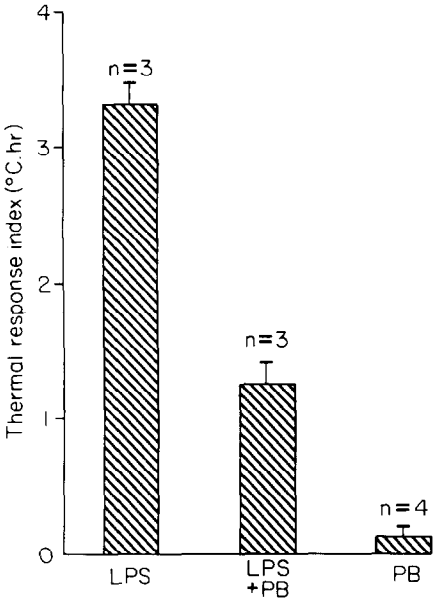

Fig. 6. Thermal response index (mean ± S.E.M.) following i.v. injection of 0.1 μg purified LPS, alone or after pre-incubation with 10 μg irradiated PB, or 10 μg irradiated PB alone. The TRI following PB alone was not significantly different from zero.

of different lots of commercially supplied LPS has been reported [21].

One explanation for the difference between our results and those of Van Miert and Van Duin is that the contaminants in crude LPS interfere with the interaction between PB and LPS, or that they are themselves pyrogenic.

Another explanation might be that some batches of PB might be pyrogenic themselves; our batches certainly seemed to be. Gamma irradiation of the PB abolished its intrinsic pyrogenicity without abolishing its ability to neutralize LPS. We presume that the irradiation destroyed a pyrogenic contaminant of PB, but we do not know what the contaminant was. It may well have been LPS itself, since the PB–LPS complex appears to retain some pyrogenicity, at least at the ratios we used (Fig. 6). Indeed, γ-radiation is known to destroy the pyrogenicity of LPS [22].

The pyrogenic action of LPS is mediated in the host by endogenous pyrogen (interleukin 1). Duff and Atkins [2] have reported the ability of PB to reduce or abolish LPS-stimulated release of endogenous pyrogen from rabbit monocytes. The PB was effective at PB:LPS ratios from 5×10^3 to 5×10^4:1. Simultaneous injection of 0.5 μg LPS and 500 μg PB (10^3:1, PB:LPS) produced fevers of latency greater than 1 hr, while LPS alone resulted in fevers with latency of 20 min. These authors do not report quantitative data on the *in vivo* pyrogenicity of PB-treated LPS.

PB binds with high affinity to lipid A [4], and we have previously shown that the lipid A moiety is responsible for the pyrogenic response of rabbits to LPS [3]. The data presented here further support the role of lipid A in the mediation of LPS-induced fever. It is plausible that PB–LPS complexes are less potent than LPS alone in releasing endogenous pyrogen from mononuclear phagocytes. The mechanism by which LPS stimulates endogenous pyrogen production is unclear. Previous reports suggest that binding of LPS to cell membranes involves the lipid A moiety of the molecule [23], so that treatment with PB may interfere with the ability of LPS to bind to target cells, thus preventing endogenus pyrogen release.

In conclusion, we have shown that pre-incubation with polymyxin B, in ratios as low as 5:1, significantly reduces the pyrogenicity in rabbits of highly purified LPS, but does not abolish it entirely.

Acknowledgements—S.J.C.W. was in receipt of a Lowenstein Research Scholarship. The authors are grateful to the South African Medical Research Council for support, through a grant to Professor Duncan Mitchell and Dr Helen Laburn.

REFERENCES

1. H. A. Bernheim, L. H. Block and E. Atkins, *Ann. intern. Med.* **91**, 261 (1979).
2. G. W. Duff and E. Atkins, *J. immun. Meth.* **52**, 33 (1982).
3. E. Kenedi, H. Laburn, D. Mitchell and F. P. Ross, *J. Physiol. Lond.* **328**, 361 (1982).
4. D. C. Morrison and D. M. Jacobs, *Immunochemistry* **13**, 813 (1976).
5. D. C. Morrison and L. Leive, *J. biol. Chem.* **250**, 2911 (1975).
6. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
7. V. S. Waravdekar and L. D. Saslaw, *J. biol. Chem.* **234**, 1945 (1959).
8. C. M. Tsai and C. E. Frasch, *Analyt. Biochem.* **119**, 115 (1982).
9. W. G. Clark and H. R. Cumby, *J. Physiol., London.* **248**, 625 (1975).
10. D. Rifkind, *J. Bacteriol.* **93**, 1463 (1967).
11. D. Rifkind and J. D. Palmer, *J. Bacteriol.* **92**, 815 (1966).
12. R. P. Gruninger and C. W. Howe, *Proc. Soc. exp. Biol. Med.* **130**, 1202 (1969).
13. E. Neter, E. A. Gorzynski, O. Westphal and O. Lüderitz, *J. Immun.* **80**, 66 (1959).
14. R. A. Fox and K. Rajaraman, *Cell Immun.* **53**, 333 (1980).
15. J. J. Carrigan Jr. and B. M. Bell, *J. Lab. clin. Med.* **77**, 802 (1971).
16. D. Rifkind and R. B. Hill Jr., *J. Immun.* **99**, 564 (1967).
17. D. M. Jacobs and D. C. Morrison, *J. Immun.* **118**, 21 (1977).
18. P. T. Mora and B. G. Young, *J. gen. Mircobiol.* **26**, 81 (1961).
19. A. S. J. P A. M. Van Miert and C. Th. M. Van Duin, *Arzneimittel-Forsch.* **28**, 2246 (1978).
20. C. Galanos, O. Lüderitz and O. Westphal, *Zeitbl. Bakt-Hyg.* **A243**, 226 (1979).
21. S. M. Wolff, J. H. Mulholland and S. B. Ward, *J. Lab. clin. Med.* **65**, 268 (1965)
22. G. Csako, R. J. Elin, D. H. Hochstein and C. M. Tsai, *Rev. Inf. Dis.* **6**, 573 (1984)
23. D. C. Morrison and R. J. Ulevitch, *Am. J. Path.* **93**, 527 (1978).