Anti-inflammatory activity of bacterial endotoxin

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Lipopolysaccharide endotoxins (LPS) from Gram-negative bacteria show anti-inflammatory activity in the kaolin-induced paw oedema test in the rat (Maguire & Wallis, 1977). We have therefore studied the effects of LPS from *E. coli* on carrageenan-induced paw oedema and in leucocyte accumulation in implanted sponges in the rat. In addition, some pharmacological properties and behaviour on sterile Sephadex columns of LPS and a fraction isolated from normal human serum have been compared.

Anti-inflammatory models. Female albino rats, 150–200 g, obtained from OLAC, 1976, were used for the carrageenan paw oedema and sponge implantation tests performed as described previously (Ford-Hutchinson, Insley & others, 1973; Walker, Smith & others, 1975b). Lipopolysaccharide B from *E. coli* 0111:B4 (Difco Laboratories, West Moseley, Surrey) was administered to each experimental group in doses ranging from 0·1 to 20 $\mu g\ kg^{-1}$ in 0·9 g/100 ml NaCl (saline) given intravenously into a tail vein immediately before either the subplantar injection of carrageenan or the insertion of the sponges. Corresponding control animals received an equal volume of saline. The local irritancy of the LPS preparation was investigated according to the directions of Atkinson, Boura & Hicks (1969). Shwartzman reactions were induced in 2 kg New Zealand white rabbits (OLAC, 1976) by the intradermal injection of 0·5 mg of the LPS preparation dissolved in 0·1 ml saline followed 24 h later by 1 mg of the LPS in 1 ml of saline given intravenously. Groups of animals each received either 1 ml of saline or 1 ml of a human plasma fraction (see below) given intravenously 1 h before the intravenous injection of the LPS. The haemorrhagic response at 4 h after the second LPS injection was assessed visually. The plasma fraction used was fraction II, made by the method of Ford-Hutchinson & others (1973), further separated on a sterile Sephadex G150 column into two fractions one of high molecular weight (>5000) and the other of low molecular weight (<5000). The columns were sterilized by autoclaving the Sephadex, buffers and all glassware at 120° for 1 h and the plastic parts by exposure to ethylene oxide for 24 h. Elution and collection procedures were made in a sterile cabinet. The high and low molecular weight fractions were tested by the carrageenan paw oedema method. In the experiments with the Shwartzman reaction the lower molecular weight material was further purified by ion exchange chromatography as described by Walker, Badcock & others (1975a).

Anti-inflammatory effects of LPS. The results in Table 1 show that *E. coli* LPS produced significant inhibitory effects on the paw swelling caused by the carrageenan and on the emigration of white cells into implanted inert sponges at a dose of 2 $\mu g\ kg^{-1}$ and above. The sponge model was more sensitive and a dose of 0·5 $\mu g\ kg^{-1}$ caused a significant reduction of the total leucocytes found in the exudate. When comparable doses of the LPS were injected directly into the paw it did not produce a local irritant action according to the criteria of Atkinson & Hicks (1971).

Anti-inflammatory effects of human plasma fraction preparations. The results in Table 2 are from a preparation of human plasma fraction, prepared by ultrafiltration followed by separation by gel filtration using a non-sterile Sephadex G25 column, and further fractionated using a sterile Sephadex G150 column. Significant anti-inflammatory activity occurred in both fractions in two separate experiments. When the lower molecular weight material was further fractionated by absorption on Sephadex A25 and elution with 5 g/100 ml acetic acid and 0·45 g/100 ml NaCl (see Walker & others, 1975a) it completely suppressed the haemorrhagic component of the local Shwartzman reaction in the rabbit.

Bacterial endotoxins possess a myriad of biological activities (Elin & Wolff, 1976) some of which are inflammatory. Thus the well-known Shwartzman reactions have been described as models of inflammatory processes resulting in severe tissue damage (Rosenthale, 1974). LPS has been shown to activate both the classical and alternative pathways of complement (Kane, May & Frank, 1973; Fine, 1974) causing changes in vascular permeability and in neutrophil chemotaxis (Mergenhagen, Snyderman & others, 1969). There is an increased release of vasoactive substances, including

Table 1. *Anti-inflammatory effects of* E. coli *LPS in the carrageenan paw oedema and sponge implantation tests in the rat.* Results are given as mean percentage reduction in experimental group in either paw volumes at 3 h or total leucocyte counts in 5 h sponge exudates compared with the corresponding control group. Those marked * show a statistically significant difference ($P < 0.005$). Each group contained at least five rats. ND—not determined.

Dose of LPS ($\mu g\ kg^{-1}$)	Paw volume	Total leucocyte count
0·1	10	18
0·5	12	39*
2·0	42*	68*
5·0	60*	82*
12·5	ND	94*
20·0	62*	ND

* Correspondence.

Table 2. *Anti-inflammatory activity of high and low molecular weight fractions from sterile Sephadex G 150.* Results are given as the mean percentage reduction in the experimental group in the paw volume at 3 h compared with the control group. All the results show a statistically significant reduction ($P < 0{\cdot}005$), each group contained five rats. Fractions are defined in the text.

Experiment	High molecular weight fraction	Low molecular weight fraction
1	47	60
2	66	37

histamine and the kinins, which may be mediated, at least in part, by activated components of the complement system (Hinshaw, Brake & Emerson, 1965). In contrast it was reported by Conti, Cluff & Scheder (1961) that non-lethal doses of LPS from *E. coli* and *Shigella flexneri* inhibited leucocyte infiltration into areas of rabbit skin infected with staphylococci. Maguire & Wallis (1977) showed that LPS from *E. coli* and *Enterobacter agglomerans* reduced the development of swelling in the kaolin paw oedema reaction in the rat. Furthermore leucocyte infiltration into the kaolin injection site was completely abolished by the subcutaneous administration of a preparation from rabbit antilymphocyte serum which contained LPS. The results of the present work (Table 1) confirm that *E. coli* LPS is a very active anti-inflammatory agent in carrageenan-induced paw oedema in the rat and at similar doses significantly inhibits the emigration of leucocytes into exudates of implanted sponges.

The mode of action may be complex. It is not mediated by a counter-irritancy mechanism since comparable amounts given by local injection did not produce an inflammatory swelling. Activation of the complement system with the subsequent formation of chemotactic factors occurs and it has been suggested that an overproduction of such factors rather than depletion of circulating complement may lead to the diminished chemotactic response in animals receiving LPS (Territo & Golde, 1976). A direct inhibitory action of LPS on circulating leucocytes cannot be excluded since intravascular fragmentation of granulocytes has been observed after LPS injections in rabbits (Horn & Collins, 1968). There is certainly a considerable degree of structural damage to the vascular endothelium (Stewart & Anderson, 1971; McGrath & Stewart, 1969; Gaynor, 1973) which may be mediated by the release of vasoactive factors and depletion of intracellular stores of these substances could contribute to an anti-inflammatory effect. Finally there is adrenocortical stimulation leading to an increased secretion of endogenous anti-inflammatory corticosteroids (Munoz, 1961).

The ability of low doses of bacterial LPS to cause anti-inflammatory effects in conventional animal models, such as paw oedemas and implanted sponges, throws doubt on the existence of some anti-inflammatory factors isolated from natural sources (Maguire & Wallis, 1977). Contamination with small amounts of endotoxin from Gram-negative bacteria may have caused spurious results. The principal site of such contamination is the various packing materials, e.g. Sephadex, used in gel chromatographic separation steps. Bacteria may grow in such columns especially in areas containing nutrients from elution media, and when lysed by the subsequent applications of hypertonic solutions will release LPS. It may then behave as a low molecular weight fraction (<5000) due to its formation on the column itself. However, on rechromatography under sterile conditions LPS will show the elution properties of its true molecular weight ($<200\,000$). In the present work (Table 2) it has been shown that when human plasma ultrafiltrate is fractionated on Sephadex under non-sterile conditions then a part of the overall anti-inflammatory activity is due to the presence of material of high molecular weight resembling LPS but that in addition a low molecular weight fraction possessing anti-inflammatory activity is also present. Further evidence that this low molecular weight material is not endotoxin is that it possesses biological properties which are antagonistic to that of *E. coli* LPS. Thus it has been shown (Walker & others, 1975a) that it will inhibit the release of chemotactic peptides and anaphylatoxin when complement is activated by *E. coli* LPS *in vitro*. In the present work the local Shwartzman reaction, elicited by LPS in the rabbit, is suppressed by intravenous administration of the low molecular weight material separated from normal human plasma.

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REFERENCES

ATKINSON, D. C., BOURA, A. L. A. & HICKS, R. (1969). *Br. J. Pharmac.*, **8**, 348–354.
ATKINSON, D. C. & HICKS, R. (1971). *Ibid.*, **41**, 480–487.
CONTI, C. R., CLUFF, L. E. & SCHEDER, E. P. (1961). *J. exp. Med.*, **113**, 845–859.
ELIN, R. J. & WOLFF, S. M. (1976). *Ann. Rev. Med.*, **27**, 127–141.
FINE, D. P. (1974). *J. Immun.*, **112**, 763–769.
FORD-HUTCHINSON, A. W., INSLEY, M. Y., ELLIOTT, P. N. C., STURGESS, E. A. & SMITH, M. J. H. (1973). *J. Pharm. Pharmac.*, **25**, 881–886.
GAYNOR, E. (1973). *Blood*, **41**, 797–807.

HINSHAW, L. B., BRAKE, C. M. & EMERSON, T. E. (1965). In: *Shock and Hypertension: Pathogenesis and Treatment*, pp. 431–441. New York: Grune & Stratton.

HORN, R. G. & COLLINS, R. D. (1968). *Lab. Invest.*, **19**, 451–459.

KANE, M. A., MAY, J. E. & FRANK, M. M. (1973). *J. clin. Invest.*, **52**, 370–376.

MAGUIRE, E. D. & WALLIS, R. B. (1977). *Br. J. Pharmac.*, **59**, 261–268.

MCGRATH, J. M. & STEWART, G. J. (1969). *J. exp. Med.*, **129**, 833–848.

MERGENHAGEN, S. E., SNYDERMAN, R., GEWURZ, H. & SHIN, H. S. (1969). *Curr. Top. Microbiol. Immun.*, **50**, 37–77.

MUNOZ, J. (1961). *J. Immun.*, **86**, 618–626.

ROSENTHALE, M. E. (1974). In: *Anti-inflammatory Agents*, II, pp. 123–192. London: Academic Press.

STEWART, G. J. & ANDERSON, M. J. (1971). *Br. J. exp. Path.*, **52**, 75–80.

TERRITO, M. C. & GOLDE, D. W. (1976). *Blood*, **47**, 539–544.

WALKER, J. R., BADCOCK, J. K., FORD-HUTCHINSON, A. W., SMITH, M. J. H. & BILLIMORIA, F. J. (1975a). *J. Pharm. Pharmac.*, **27**, 747–753.

WALKER, J. R., SMITH, M. J. H., FORD-HUTCHINSON, A. W. & BILLIMORIA, F. J. (1975b). *Nature*, **254**, 444–446.

Penicillins and the limulus amoebocyte lysate test for endotoxin

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The use of the limulus amoebocyte lysate test for the detection of pyrogens has recently been reviewed by Cooper (1975). Eibert (1972) reported that 84 samples of injectable drugs gave consistent results when tested by the official rabbit test or the limulus test. Nguyen & Greppin (1974), using a nephelometric adaptation of the test of Eibert reported that a number of injectable products inhibited the reaction with limulus amoebocyte lysate. I have used a technique similar to that described by Eibert to establish (a) the causes and extent of penicillin inhibition of the limulus test, and (b) the usefulness of the test with penicillins compared with the official rabbit pyrogen test.

Limulus polyphemus amoebocyte lysate, as 0·1 ml limulus reagent from Mallinckrodt Chemical Co., or from Associates of Cape Cod Inc. was added to 0·1 ml of test solution in 7 mm diameter glass test tubes and incubated for 60 min at 37°. Tubes were then gently inverted and read as follows: positive (+) = firm gel adhering to bottom of tube; negative reaction (−) = clear solution with no gelation, running freely from tube; intermediate reaction (±) = viscous or flocculent tube contents deforming on inversion of tube. Positive (endotoxin in water) and negative (water for injection) controls were run in parallel with all tests.

S. minnesota R595 glycolipid endotoxin was prepared according to Galanos, Luderitz & Westphal (1969). Purified *E. coli* lipopolysaccharide was obtained from Associates of Cape Cod Inc. Benzyl penicilloate, BRL 1071 and Penicillin G polymer were prepared in these laboratories according to Clark, Johnson & Robinson (1949), Doyle & Nayler (1961) and Smith & Marshall (1971). Other materials were from commercial sources.

The effect of penicillins at various concentrations on the sensitivity of the limulus test for a purified endotoxin obtained from *S. minnesota* was examined. Results for methicillin (Table 1) showed that in the concentration range 0–10 mg ml^{-1} methicillin had no effect on the gelation reaction, so that the sensitivity of that test for pyrogen in the antibiotic could be increased by increasing the antibiotic concentration. Above 10 mg ml^{-1} methicillin inhibited the limulus gelation reaction such that the sensitivity of the test decreased with increasing antibiotic concentration. For subsequent limulus tests the optimum concentration of methicillin (10 mg ml^{-1}) was used. Qualitatively similar results were obtained with other penicillins, but the highest concentration of antibiotic that failed to inhibit gelation (i.e. the optimum test concentration) varied with the structure of the penicillin; the optimum test concentrations and levels of