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Plasma acid phosphatase levels in endotoxaemia: modification by drugs and chemically detoxified endotoxins

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1 The ability of chemically detoxified *E. coli* endotoxins and membrane-active agents to modify the toxicity of native *E. coli* endotoxin *in vivo* was examined.

2 The time- and dose-dependent increase in plasma acid phosphatase activity following toxin administration to rats provided a convenient quantitative measure of *in vivo* toxicity under various experimental conditions.

3 Treatment of endotoxin with either sodium hydroxide or sodium periodate produced substances which, when injected alone, failed to cause an increase in plasma acid phosphatase activity. When given before native endotoxin, periodate-detoxified toxin produced a dose-dependent reduction in the elevation of plasma enzyme activity caused by unmodified toxin. Pretreatment with pranolium, hydrocortisone or (+)-propranolol also reduced the *in vivo* toxicity of endotoxin.

4 Mortality studies in mice provided further independent support for the effectiveness of periodate-detoxified endotoxin and membrane-active drugs as endotoxin antagonists.

5 Evidence has been found that under certain conditions gentamicin may act synergistically with bacterial endotoxins *in vivo*.

Introduction

Endotoxin release from gram-negative bacteria is believed to play a major role in the pathogenesis of gram-negative septic shock (Caridis, Reinhold & Woodruff, 1972; Woodruff, O'Carroll & Koizumi, 1973). Studies of the biological effects of these macromolecular lipopolysaccharide complexes have shown that these substances are capable of exerting actions on a wide variety of physiological processes (Kass & Wolff, 1973). This diversity of effects has greatly complicated both efforts to elucidate the molecular basis of endotoxic shock and also the therapeutic management of this life-threatening complication of gram-negative septicaemia. Despite aggressive antibiotic therapy (usually involving the aminoglycosides), the mortality of patients with gram-negative septic shock is extremely high and this may be largely attributed to the persistent actions of endotoxins derived from the cell walls of the infecting gram-negative bacterium. The clinical management of septic shock might be greatly improved if specific antibiotic therapy could be combined with agents capable of antagonizing the actions of endotoxin *in vivo*.

Bacterial endotoxins are lipophilic complexes capable of interacting with and perturbing natural and artificial membrane systems (Davies, Stewart-Tull & Jackson, 1978; Esser & Russell, 1979; Onji & Liu, 1979; Godin, Tuchek & Garnett, 1982). Such actions *in vivo* could lead to altered cellular homeostasis and, eventually, to cell death, with the release of cellular components, including lysosomal hydrolases, whose levels in plasma are known to increase following endotoxin administration (Janoff, Weissmann, Zweifach & Thomas, 1962). If cell membrane disruption is a major determinant of endotoxin action, amphipathic molecules with membrane stabilizing properties might be expected to exert beneficial actions in endotoxaemia. Such a possibility is suggested by recent studies involving the haemolytic streptococcal exotoxin streptolysin O, the reduced form of which is haemolytic and potentially lethal. Pretreatment of mice with chlorpromazine, a prototype membrane stabilizer, affords protection against toxin-induced mortality and chlorpromazine is also effective in protecting against the haemolytic actions of the toxin *in vitro* (Besluau, Gauthier-Rohman &

Halpern, 1979). Membrane stabilizers, therefore, may potentially represent one class of endotoxin antagonist.

Endotoxin actions *in vivo* may also involve binding to structurally selective lipopolysaccharide receptors, such as that recently described in the erythrocyte membrane (Yokoyama, Terao & Osawa, 1978). Interactions at this level might be effectively antagonized by chemically-modified toxins devoid of lethality but still capable of competing with native toxin at specific binding sites on target tissues.

The aim of the present study was to assess the ability of membrane-active drugs and chemically-detoxified endotoxins to modify the actions of subsequently administered native toxin, using elevations in plasma acid phosphatase activity as a convenient index of endotoxin toxicity *in vivo*.

Methods

Male Wistar rats (200–350 g) were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, intraperitoneally). *E. coli* endotoxin (026:B6, Difco) was suspended in distilled water and sonicated on ice for 2 min at 50 W power with a Braunsonic 1510 sonicator before injection. Sonication was used to produce a homogeneous suspension of endotoxin to ensure reproducible efficacy. Endotoxin was infused at a dose of 10 mg kg^{-1} over a 45 s period via a catheter inserted into the jugular vein. Plasma samples were obtained at various times following endotoxin administration by withdrawing 1.0 ml blood from a catheter placed in the right carotid artery into a heparinized syringe. In each case, animals were given 1.0 ml saline to replace the volume of blood removed. Blood was immediately centrifuged and the plasma analyzed for acid phosphatase activity as described below.

Detoxified endotoxin derivatives were prepared by treating native toxin with sodium hydroxide or sodium periodate as described by Neter, Westphal, Luderitz, Gorynski & Eichenberger (1956). These chemically-modified endotoxins were administered intravenously 30 min before challenge with native endotoxin (10 mg kg^{-1}). The effects of pretreating rats with membrane-active drugs 10 min before endotoxin administration were also examined. The agents tested (at the dose indicated) were chlorpromazine (Poulenc Ltd., Montreal, Quebec) (0.25 mg kg^{-1}), (+)-propranolol (Ayerst Laboratories) (0.1 mg kg^{-1}), pranolium (N,N-dimethyl propranolol) chloride (G.D. Searle and Co.) (0.20 mg kg^{-1}), and hydrocortisone sodium succinate (Sigma) (35 mg kg^{-1}). The doses of the drugs employed produced minimal alterations in blood pressure and were selected largely on the basis of clinical usage.

Acid phosphatase measurements were performed on diluted plasma samples (1:5 in distilled water) as follows. An aliquot of diluted plasma (0.2 ml) was incubated with 0.5 ml citrate buffer (0.09 M, pH 4.8) and 0.5 ml *p*-nitrophenylphosphate (Sigma) solution (4 mg/ml) at 37°C for 30 min. The reaction was terminated with 5.0 ml 0.1 N NaOH and the concentration of liberated *p*-nitrophenol was determined spectrophotometrically by measuring the absorbance at 410 mu. Activities were expressed as Sigma units/ml plasma (Sigma technical bulletin, number 104).

Preliminary experiments were undertaken to assess the possible protective effects of membrane-active drugs or chemically-detoxified endotoxins on endotoxin lethality. Swiss Albino white mice rather than rats were used in these studies because of the relatively large numbers of animals required. Evaluation of relative drug efficacies was complicated by the rather protracted time course of the observation period (up to 24 h) in combination with uncertainties regarding the pharmacokinetic properties of the drugs in question under the particular experimental conditions employed. The data presented in Table 1 represent the results of our initial attempts to optimize drug administration in order to obtain maximal protective effects. All drugs were given in saline by intraperitoneal injection. Detoxified toxins were administered as a single dose (20 or 40 mg kg^{-1}) 1 h before native endotoxin. Hydrocortisone (35 mg kg^{-1}) and pranolium (0.5 mg kg^{-1}) were given 1 h before toxin and a second dose 12 h later. Animals treated with (+)-propranolol received a 0.25 mg kg^{-1} dose 30 min before endotoxin and every 6 h up to 18 h. Native endotoxin was administered at a dose of 40 mg kg^{-1} by intraperitoneal injection.

In the experiments studying the effects of gentamicin on endotoxin toxicity, rats were pretreated with the antibiotic either acutely (1 or 10 mg kg^{-1} intravenously, 15 min before endotoxin challenge) or chronically (1 mg kg^{-1} daily for three days, given as twice daily intraperitoneal injections of 0.5 mg kg^{-1}). Neither of these modes of gentamicin administration had any effect on levels of plasma acid phosphatase. Endotoxin was then administered intravenously at a dose of 10 mg kg^{-1} and plasma acid phosphatase activities measured 3 h later.

Assessment of statistical significance was made in all cases by Student's *t* test.

Results

The marked effect of native endotoxin on plasma acid phosphatase activity in the rat at various times following toxin administration is illustrated in Figure 1. At the particular dose of endotoxin employed (10 mg kg^{-1}) most animals did not survive appreci-

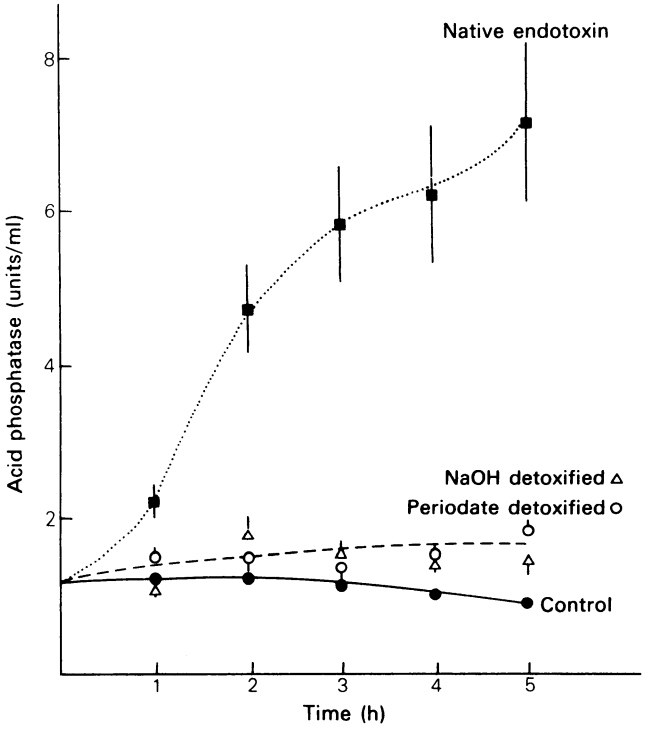

Figure 1 Effect of native (unmodified) and detoxified *E. coli* endotoxins (10 mg kg^{-1}) on plasma acid phosphatase elevations in the rat. Each experimental group consisted of 5 animals except the native endotoxin-treated and control group which contained 10 animals. The values (mean with s.e.mean shown by vertical lines) for these two latter groups presented in Figures 1–3 represent pooled data from various independent measurements made during the course of the experiments depicted in these figures.

ably longer than the final indicated time of 5 h and a highly statistically significant ($P < 0.05$) elevation in plasma acid phosphatase activity was present as early as 1 h following endotoxin administration. Figure 1 also indicates that treatment of native endotoxin with either sodium hydroxide or sodium periodate virtually abolished the ability of the modified lipopolysaccharide complex to elevate plasma acid phosphatase levels. Control animals, which were serially bled with saline replacement as in the experimental group, did not exhibit increases in acid phosphatase activity. Further, measurements of plasma acid phosphatase levels at 3 h following endotoxin administration in serially bled animals and animals from which a single blood sample was taken were indistinguishable. Therefore, it seems unlikely that the blood sampling procedure influenced measured enzyme levels in our experimental model although it is appreciated that circulating plasma levels and factors other than those enzymes measured, may be complicated by the blood loss in the present protocol.

Figure 2 illustrates the effects of pretreatment with chemically detoxified endotoxins on the elevation in plasma acid phosphatase levels produced by subsequently administered native endotoxin. At a dose of 2.5 mg kg^{-1}, sodium hydroxide-detoxified and sodium periodate-detoxified (data not shown) toxins produced equivalent reductions in acid phosphatase activities but these did not attain statistical significance. However, 5.0 mg kg^{-1} periodate-modified toxin caused highly significant ($P < 0.01$) decreases in plasma enzyme activity which were apparent by 2 h after native toxin injection. Increasing dosage of sodium hydroxide-detoxified endotoxin to 5 or 10 mg kg^{-1} failed to reveal significant protective effects (Figure 2).

The effects of pretreatment with various pharmacological agents possessing membrane-stabilizing properties on endotoxin-induced elevations in plasma acid phosphatase activity were next examined (Figure 3). While all the drugs studied showed some tendency to reduce plasma enzyme levels, (+)-propranolol exerted the greatest protective effects which were highly significant ($P < 0.02$) from 2 h onward. The decreases produced by pranolium and hydrocortisone were significant ($P < 0.05$) only at 2 and 5 h, respectively (Figure 3). It should be noted that none of the pharmacological agents tested af-

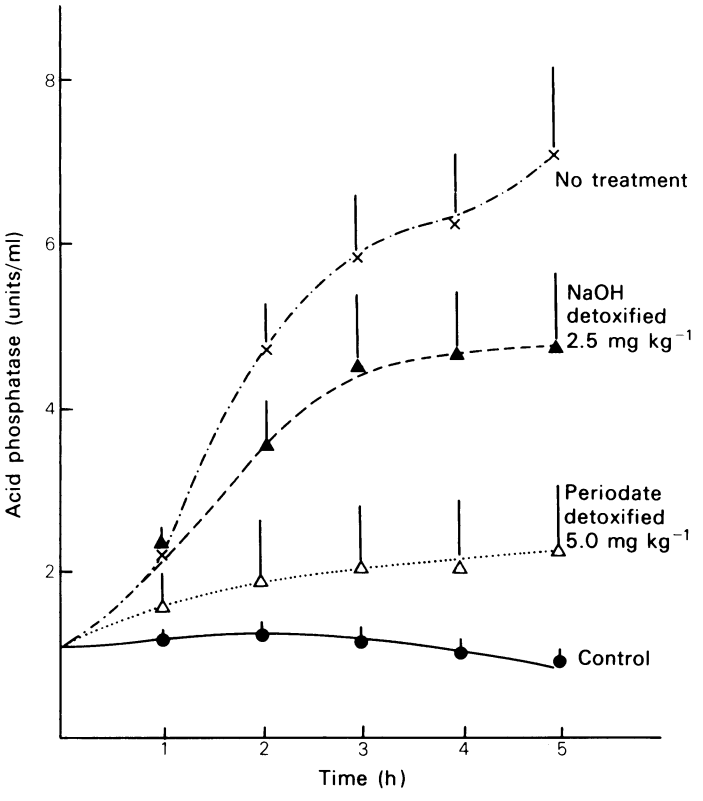

Figure 2 Effects of pretreatment with periodate-detoxified or sodium hydroxide-detoxified endotoxin on the increase in plasma acid phosphatase activity resulting from native toxin (10 mg kg^{-1}) administration. Each point represents mean with s.e.mean shown by vertical lines. Each experimental group consisted of 5 animals except the no treatment group which contained 10 animals.

fected the activity of plasma acid phosphatase when tested *in vitro* at the highest concentrations attainable in plasma at their respective doses assuming a blood volume equal to 7% of body weight.

Having thus demonstrated that periodate-detoxified endotoxin and certain membrane-active substances are capable of modifying endotoxin action *in vivo*, the question arose as to the relationship between reductions in plasma acid phosphatase activities and other manifestations of endotoxin toxicity, the ultimate one being death. We have, therefore, undertaken preliminary experiments in mice to determine whether the compounds shown here to behave as endotoxin antagonists on the basis of plasma acid phosphatase measurements are also able to reduce the lethality of endotoxin when administered *in vivo*. Our results (summarized in Table 1) provide evidence that these substances are indeed capable of reducing or at least delaying mortality due to endotoxin. The fact that protection was greatest at the earlier observation times is probably referable to the dose regimens employed (see Methods). Thus, drugs were given as an initial dose before endotoxin challenge and this was repeated at 12 h (in the case of hydrocortisone and pranolium) or at 6, 12 and 18 h for (+)-propranolol. It is also worth noting that the protective effect at 12 h of periodate-detoxified endotoxin, given as a single dose before endotoxin, had virtually disappeared by 18 h. Complexities in the dose-dependence of protection by detoxified toxins were revealed by our observation that an increase in the administered dose of periodate-detoxified toxin from 20 to 40 mg kg^{-1} was associated with a reduction in its protective actions (mortalities at 12 and 18 h were increased to 20 and 80%, respectively).

In view of the extensive use of aminoglycoside antibiotics in the management of gram-negative septicaemia clinically, it was decided to apply the approaches described here to an investigation of the possible influence of gentamicin therapy on the toxicity of *E. coli* endotoxin *in vivo*. When administered acutely to rats at a dose of 1 mg kg^{-1} intraveneously,

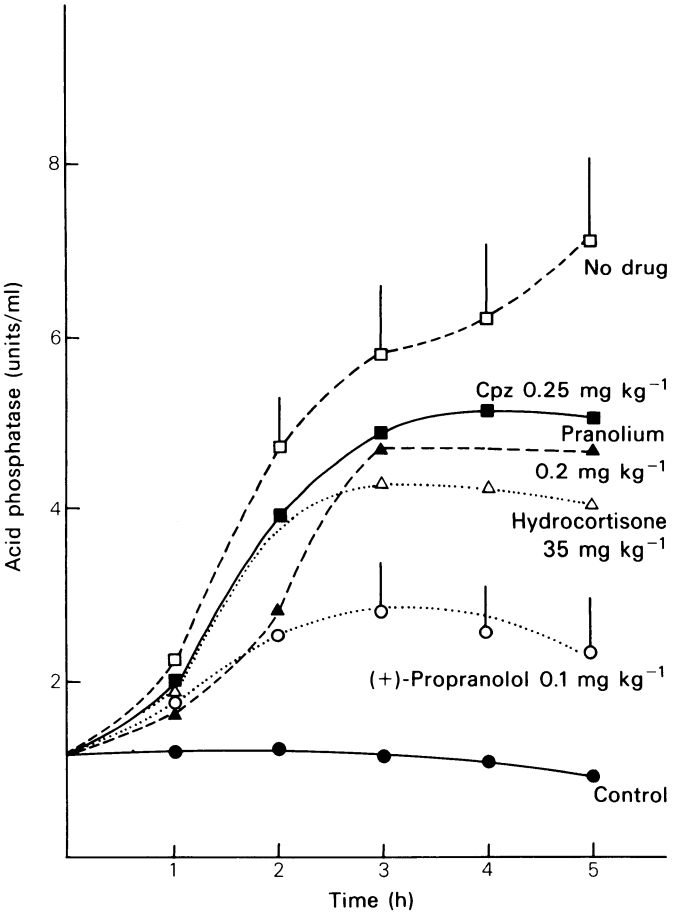

Figure 3 Effect of pretreatment with various membrane-active agents on the increase in plasma acid phosphatase activity resulting from native toxin (10 mg $^{-1}$) administration. Each point represents mean with s.e.mean shown by vertical lines. The drug-treated groups contained 5 animals. Cpz = chlorpromazine.

gentamicin caused a slight, although not statistically significant, increase in the plasma acid phosphatase activity elicited by endotoxin administration (Figure 4). However, a pronounced elevation in enzyme levels ($P < 0.01$) was seen after endotoxin administration both in animals subjected to chronic gentamicin therapy (1 mg kg^{-1} daily, intraperitoneally, for three days) and in animals given a single high dose of gentamicin (10 mg kg^{-1} intravenously) (Figure 4).

Discussion

It has been well documented in experimental studies of gram-negative sepsis that mortality cannot be prevented by antibiotics alone (Greisman, Du Buy & Woodward, 1979; Hinshaw, Archer, Beller-Todd, Coalson, Flournoy, Parsey, Benjamin & White, 1980). Further, in a recent report of a large number of patients with gram-negative bacteraemia, it has been shown that antecedent antibiotic treatment may actually be associated with a significant increase in the frequency of shock and that under certain conditions fatality rates may be unfavourably influenced by gentamicin therapy (Kreger, Craven & McCabe, 1980). These considerations, when taken with our own preliminary findings indicating that gentamicin appears capable of acting synergistically with endotoxin *in vivo* (Figure 4), emphasize the need for therapeutic measures aimed at antagonizing the effects of endotoxin in the management of gram-negative sepsis.

Increased levels of lysosomal enzymes in the blood have long been known to be associated with endotoxaemia both in man and in experimental animals (Janoff *et al.*, 1962; Sardesai & Rosenberg, 1974).

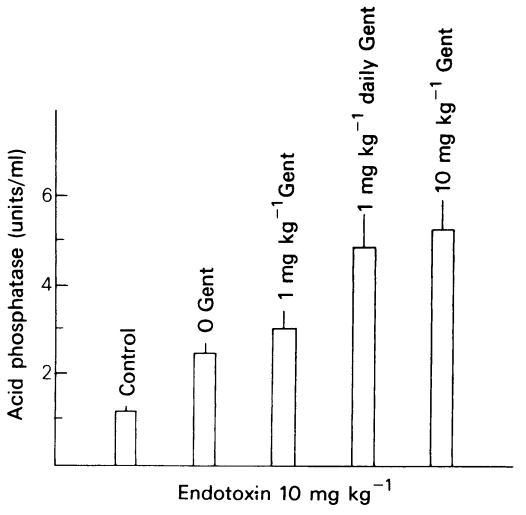

Figure 4 Effect of acute (1 mg kg^{-1} or 10 mg kg^{-1}) and chronic (1 mg kg^{-1} daily for three days) gentamicin (Gent) pretreatment on the activity of plasma acid phosphatase 3 h after the injection of native endotoxin (10 mg kg^{-1}). Each value represents the mean of experiments using 10 animals per group; s.e.mean shown by vertical lines. Plasma acid phosphatase levels in the animals receiving gentamicin chronically or at a dose of 10 mg kg^{-1} acutely were significantly ($P<0.01$) greater than those in endotoxin-treated animals not receiving gentamicin.

While the release of these lysosomal hydrolases is presumed to be a reflection of cellular injury (Janoff *et al.*, 1962), these enzymes may also contribute to the organ damage occurring in experimental endotoxaemia or clinical septicaemia (De Palma, Coil, Davis & Holden, 1967; Caridis *et al.*, 1972). The major sources of the lysosomal hydrolases are believed to be splanchnic viscera (especially the liver and pancreas) as well as leucocytes sequestered in pulmonary capillaries (Lefer, 1976; Demling, Proctor, Grossman, Duy & Starling, 1981). The observed elevation in plasma lysosomal hydrolase activities in endotoxaemia is probably the result of a complex interplay between an enhanced release from damaged cells and a reduction in plasma clearance (Spath, Reed & Lefer, 1975). Thus, the effects of pharmacological interventions on plasma enzyme levels and, ultimately, delaying mortality must be considered in the light of these two distinct processes.

The disruption of plasma membrane integrity is undoubtedly a crucial event governing the release of lysosomal enzymes into the circulation following endotoxin administration. Gram-negative bacterial endotoxins have been shown to interact with and perturb a variety of natural and artificial membrane systems (Davies *et al.*, 1978; Onji & Liu, 1979; Esser & Russell, 1979; Godin *et al.*, 1982). The possible involvement of membrane changes early in endotoxaemia is suggested by the results of a recent study using skeletal muscle membrane potential as an index of cellular alterations induced by endotoxin and showing that membrane depolarization (triggered by changes in Na^+ and K^+ distribution) preceded the development of deep hypotension and cellular ATP depletion (Illner & Shires, 1981). Our observation that some membrane-active agents are effective in reducing the *in vivo* toxicity (Figure 3) and delaying mortality (Table 1) due to *E. coli* endotoxin may reflect the ability of these substances to stabilize plasma membranes of endotoxin-sensitive target tissues in a manner analagous to that involved in their antihaemolytic actions (Seeman, 1972). The beneficial effects of corticosteroids such as methylprednisolone (Hinshaw *et al.*, 1980; Brigham, Bowers & McKeen, 1981) and of lidocaine (Fletcher & Ramwell, 1978) in experimental endotoxaemia further support the concept that drugs with membrane stabilizing properties may act as endotoxin antagonists *in vivo*. Of the various amphipathic substances tested here, the protective effects of (+)-propranolol both on plasma acid phosphatase elevation (Figure 3) and on mortality (Table 1) seemed to be the most marked. This is unlikely to arise from a greater efficacy of propranolol as a membrane stabilizer, propranolol and pranolium being quite equivalent in this regard (Godin, Au & Garnett, 1979), but may reflect the tendency of propranolol to become concentrated in the lung (Hayes & Cooper, 1971; Schneck, Pritchard & Hayes, 1977), a tissue which is

Table 1 Effects of drug- and detoxified endotoxin-pretreatment on the lethality of *E. coli* endotoxin in mice.

Treatment	*% mortality*		
	12 h	*18 h*	*24 h*
None* ($n=20$)	28±8	78±8	98±2
Hydrocortisone (35 mg kg^{-1}) ($n=10$)	10	30	80
Pranolium (0.5 mg kg^{-1}) ($n=10$)	0	30	80
(+)-Propranolol (0.25 mg kg^{-1}) ($n=20$)	0	15	80
Periodate-detoxified endotoxin (20 mg kg^{-1})	10	70	100

E. coli endotoxin (serotype 026:B6, lot number 623122 Difco) was used at a dose of 40 mg kg^{-1}. Pretreatment regimens are given in the Methods section.

*Control values are given as the mean±s.d. of results obtained with three groups of animals ($n=20$, $n=20$ and $n=10$) challenged with native (unmodified) endotoxin.

highly vulnerable to the effects of endotoxin (Kaplan, Sahn & Petty, 1979).

Drugs may influence endotoxin toxicity by modifying its distributional characteristics *in vivo* (Mathison, Ulevich, Fletcher & Cochrane, 1980). Thus, for example, dexamethasone has been shown to decrease the accumulation of endotoxin complexes in the adrenal gland (Munford, Andersen & Dietschy, 1981). The physicochemical properties of bacterial endotoxins are known to be modified *in vivo* as the result of interaction with plasma components such as high density lipoproteins (Ulevitch, Johnson & Weinstein, 1979) or the lipopolysaccharide inactivator protein described by Johnson, Ward, Goralnick & Osborn (1977). Drugs might well be capable of inducing marked alterations in the biological activity and tissue distribution of endotoxin by interfering with such interactions in the plasma. Alternatively, lipophilic drugs may act by binding directly to the lipid A component of the endotoxin molecule, as has recently been postulated in the case of polymyxin B (Hughes, Madan & Parratt, 1981).

The results of our experiments with chemically-modified endotoxins, which are devoid of lethality and the ability to cause increases in plasma acid phosphatase activity (Figure 1), have shown that periodate-detoxified (Figure 2) toxin is able to reduce the effects of native endotoxin *in vivo*. However, two major limitations are apparent. First, this substance has a plasma half-life of approximately 2 h (Godin & Tuckek, unpublished observations), presumably reflecting a high rate of hepatic clearance (Utili, Abernathy & Zimmerman, 1977) and probably necessitating repeated or continuous administration for optimal endotoxin antagonism (Table 1). Secondly, our observation of complexities in concentration-dependence with decreased protection at high doses may reflect an interplay between beneficial effects resulting from endotoxin antagonism at critical sites of action and detrimental effects which may involve displacement of native toxin from tissue or plasma binding sites and/or interference with mechanisms involved in the clearance or detoxification of unmodified endotoxin *in vivo*. These considerations may well explain our inability to demonstrate significant beneficial effects of sodium hydroxide-detoxified toxin. Thus, while limitations in the quantities of detoxified endotoxins available precluded more detailed studies of their effectiveness as endotoxin antagonists *in vivo*, our results to date have led us to focus our future research efforts on exploring the molecular actions of the structurally simpler and apparently more efficacious amphipathic membrane-active agents such as propranolol in experimental endotoxaemia.

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