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In vivo and *in vitro* studies of bacterial endotoxin-membrane interactions and the effects of membrane-active agents

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1 The characteristics of ^{51}Cr-labelled *E. coli* endotoxin binding to human erythrocyte membranes *in vitro* have been investigated. A saturable component of binding was apparent at low endotoxin concentrations ($< 50\ \mu g\ ml^{-1}$) relevant to its *in vivo* actions, while at higher concentrations binding was non-saturable and increased in linear fashion. Experiments examining the ability of unlabelled endotoxin to antagonize the binding of labelled toxin provided further evidence for these specific and non-specific modes of endotoxin-membrane interaction.

2 Membrane-active agents previously shown to reduce endotoxin toxicity *in vivo* decreased endotoxin binding to erythrocyte membranes *in vitro*, with propranolol and pranolium being the most effective in this regard.

3 Tissue distribution studies following administration of radiolabelled endotoxin to guinea-pigs showed a positive correlation between the accumulation of ^{51}Cr-endotoxin in lung and elevations in plasma acid phosphatase activity, a measure of *in vivo* endotoxin toxicity.

4 The *in vivo* accumulation of ^{51}Cr-endotoxin in guinea-pig lung was reduced by prior treatment with (+)-propranolol or pranolium, paralleling the results of the *in vitro* binding studies.

5 Our results suggest that membrane-active agents such as (+)-propranolol may be useful adjuncts to antimicrobial drugs in the therapy of gram-negative endotoxaemia.

Introduction

Gram-negative bacteraemia continues to be associated with a high degree of mortality and morbidity despite the availability of powerful antimicrobial agents, such as the aminoglycosides, and of refined life-support and monitoring systems (Wolff, 1982). A major factor which complicates the therapeutic management of this clinical condition is the release from bacterial cell walls of biologically active lipopolysaccharide complexes (or endotoxins) which have been implicated in the pathogenesis of gram-negative sepsis and shock (Caridis *et al.*, 1972; Kass & Wolff, 1973; Young *et al.*, 1977). Conventional pharmacological agents employed under these conditions leave the actions of these endotoxin components unopposed and, indeed, there are indications that aminoglycosides may, under certain conditions, lead to an enhancement of their toxic potential (Kreger *et al.*, 1980; Godin & Tuchek, 1983). Substances capable of antagonizing, either directly or indirectly, the deleterious actions of bacterial endotoxins should, in combination with appropriate antimicrobial and other therapeutic measures, be of great value in the treatment of patients with gram-negative bacteraemia.

Recent studies by Ziegler *et al.* (1982) have demonstrated in patients with gram-negative bacteraemia the beneficial effect of antisera containing antibodies directed against the core elements of the endotoxin complex, including the lipid A component which is thought to be the primary toxic moiety. Another possible approach is suggested by the known ability of bacterial endotoxin to interact with and perturb cellular membranes (Davies *et al.*, 1978; Esser & Russell, 1979; Godin *et al.*, 1982), thereby suggesting the study of amphipathic membrane-active drugs as potential endotoxin antagonists. Such substances might interfere with endotoxin-membrane interaction and/or decrease the disruptive consequences of such interaction by virtue of their 'membrane-stabilizing' effects (Seeman, 1972). Consistent with this suggestion is the observation that lidocaine infusion ($1\ mg\ kg^{-1}\ h^{-1}$) increased the sur-

[1] Correspondence

vival of dogs given *E. coli* endotoxin (1 mg kg^{-1}) from 40% in untreated animals to 100% following lidocaine treatment (Fletcher & Ramwell, 1978). Methylprednisolone either alone (White *et al.*, 1978) or in combination with gentamycin (Hinshaw *et al.*, 1980) has been shown to influence favourably the course of experimental endotoxaemia and one component of this beneficial effect may be referable to the membrane actions of this steroidal agent. Recent studies in our own laboratory have demonstrated that pretreatment of rats with hydrocortisone, (+)-propranolol or pranolium resulted in significant protection against *E. coli* endotoxin-induced elevations in plasma lysosomal hydrolase activity and mortality rate, with (+)-propranolol exhibiting a particularly marked effect (Godin & Tuchek, 1983).

In the present investigation, we have attempted to derive information regarding the molecular basis by which membrane-active agents such as propranolol exert their protective actions and the results obtained suggest that propranolol is capable of acting as an effective endotoxin antagonist *in vivo*.

Methods

Binding of ^{51}Cr-labelled E. coli *endotoxin to erythrocyte membranes* in vitro

Purified *E. coli* endotoxin (Difco, serotype 026: B6) was labelled with ^{51}Cr (obtained as chromic chloride in 0.5 N HCl from New England Nuclear) as described by Braude *et al.* (1955). Binding of labelled toxin to haemoglobin-free erythrocyte membranes prepared by stepwise hypotonic lysis (Godin & Schrier, 1970) was measured by a slight modification of a procedure for intact erythrocytes (Godin *et al.*, 1982) where the membranes were first washed twice with 0.9% NaCl–15 mM Tris, pH 7.0, and resuspended in the same buffer medium to a protein concentration of approximately 0.6 mg ml^{-1}. An aliquot (0.5 ml) of this membrane suspension was then used for endotoxin binding measurements as previously described for intact erythrocytes, except for the omission of the H_2O_2 bleaching step prior to scintillation counting (Godin *et al.*, 1982). The binding data were analysed using a NONLIN curve fitting programme, as described by Al-Jobore & Roufogalis (1981). For displacement studies, human erythrocyte membranes (prepared as above) were preincubated with propranolol (HCl salt, Sigma), pranolium (chloride salt, G.D. Searle and Co.), lidocaine (HCl salt, Sterilab) or methylprednisolone (sodium succinate, Upjohn) for 5 min at 37°C. Binding of endotoxin was then initiated by the addition of ^{51}Cr-labelled toxin at a final concentration of 25 μg ml^{-1} and incubation of the mixture for 15 min at 37°C. The degree of ^{51}Cr-endotoxin binding was then assessed as described above.

Distribution of ^{51}Cr-labelled E. coli *endotoxin* in vivo

Guinea-pigs were anaesthetized with urethane (1000 mg kg^{-1}, intraperitoneally) and the right jugular vein catheterized for the injection of ^{51}Cr-labelled endotoxin. Three hours following the administration of toxin (at doses of 1, 3, or 6 mg kg^{-1}) blood samples were taken for the measurement of plasma acid phosphatase activity (Godin & Tuchek, 1983). Animals were then killed and samples of liver, spleen, lung, kidney and heart tissue were taken for the measurement of ^{51}Cr-labelled toxin content. Tissues were prepared for scintillation counting by adding 1.0 ml Protosol (New England Nuclear) to 50 mg blotted tissue in a scintillation vial and digesting for several hours at 55°C to achieve complete solubilization. Samples were cooled, decolourized with 0.1 ml 30% hydrogen peroxide, incubated another 30 min at 55°C and allowed to cool. Ten ml Econofluor (New England Nuclear) were added to the vial and radioactivity was determined by scintillation counting (using the ^{3}H channel) after 1 h of dark and temperature (10°C) equilibration. ^{51}Cr-endotoxin accumulation was expressed in ng mg^{-1} tissue wet weight. Effects of drug pre-treatment on the accumulation of ^{51}-Cr-labelled toxin in lung tissue were examined by ad-

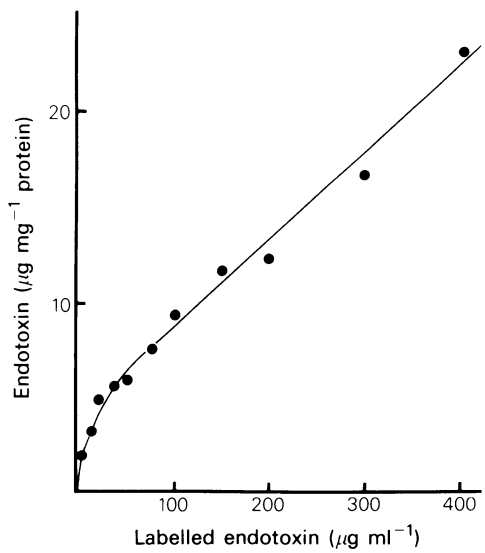

Figure 1 ^{51}Cr-*E. coli* endotoxin binding to human erythrocyte membranes. The curve through the experimental points was obtained by a non-linear curve fitting programme based on a model involving saturable and non-saturable components of binding (Al-Jobore & Roufogalis, 1981).

ministering various pharmacological agents intravenously 10 min prior to the injection of labelled toxin (3.0 mg kg^{-1}). Three hours later, animals were killed and the ^{51}Cr content of the lung tissue was determined as described above. Methylprednisolone (35 mg kg^{-1}), pranolium (0.1 mg kg^{-1}) and (+)-propranolol (Ayerst Laboratories, 0.1 mg kg^{-1}) were given as single intravenous injections while lidocaine was administered as a continuous intravenous infusion (1 mg $kg^{-1} h^{-1}$).

Results

Human erythrocytes provided a convenient model system with which to investigate the association of *E. coli* endotoxin with membranes and the effects of various pharmacological agents on this interaction. Haemoglobin-free erythrocyte membranes bound the ^{51}Cr-labelled endotoxin in a concentration-dependent manner which suggested the existence of saturable high affinity binding at low endotoxin concentrations and a linear non-saturating component at high concentrations (Figure 1). This was confirmed by non-linear least squares analysis using the following equation:

$$[\text{bound toxin}] = \frac{[R_t] \times [\text{toxin}]}{K_d + [\text{toxin}]} + C[\text{toxin}]$$

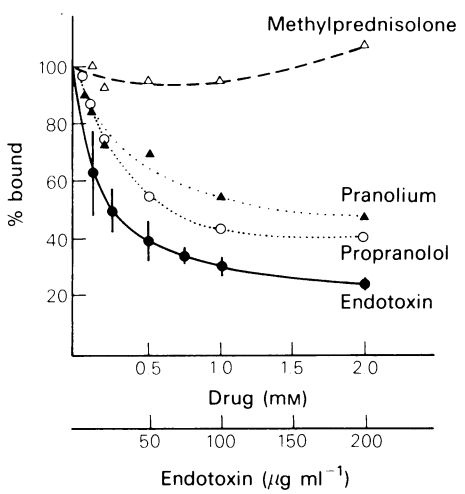

Figure 2 Effect of various concentrations (0–2.0 mM) of methylprednisolone, pranolium and (+)-propranolol on the displacement of ^{51}Cr-endotoxin bound to human erythrocyte membranes is compared to unlabelled *E. coli* endotoxin. Results are expressed as a percentage of the amount of ^{51}Cr-endotoxin bound in the absence of drug or unlabelled toxin (100% bound). Concentration of ^{51}Cr-endotoxin was 25 μg ml^{-1}. Data represent the mean with s.d. of 3 separate experiments (s.d. for drugs not shown for sake of clarity).

where $[R_t]$ is the total number of specific toxin binding sites, K_d is the dissociation constant of toxin for the saturable site and C is a constant describing the linear non-saturating component of binding. The computer-generated curve for the experimental points is that shown in Figure 1 (correlation coefficient $r = 0.995$), with $[R_t] = 4.73$ μg endotoxin mg^{-1} membrane protein, $K_d = 8.90$ μg ml^{-1} and $C = 0.44$. Since blood concentrations of endotoxin in excess of 50 μg ml^{-1} are rarely encountered, even in experimental endotoxaemia, it seems likely that the interactions involving the saturable site in our model membrane system are the most relevant to the *in vivo* actions of endotoxin. The binding of ^{51}Cr-labelled endotoxin (25 μg ml^{-1}) at this specific high affinity site was found to be displaceable by unlabelled toxin to the extent of approximately 75% (Figure 2).

The ability of several pharmacological agents to influence the binding of *E. coli* endotoxin to erythrocyte membranes was next examined (Figures 2 and 3). Of the various agents tested, propranolol and pranolium were the most effective in reducing binding, lidocaine produced a small decrease and methylprednisolone showed a tendency to enhance toxin binding at the highest concentration tested.

As an extension of the foregoing *in vitro* observations, we have investigated the possibility that propranolol may also be capable of acting as an effective endotoxin antagonist *in vivo*. To this end, preliminary experiments were performed to determine the tissue distribution of ^{51}Cr-labelled endotoxin 3 h after its

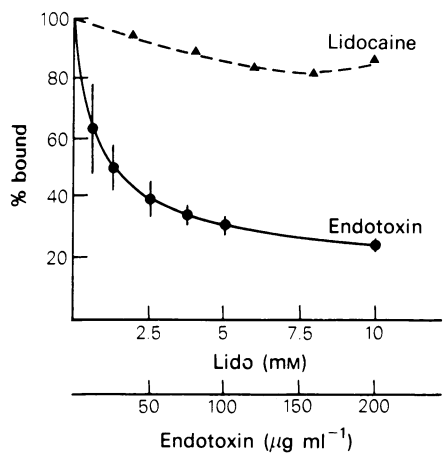

Figure 3 Ability of lidocaine to displace ^{51}Cr-*E. coli* endotoxin from human erythrocyte ghosts is compared to the displacement profile obtained with unlabelled *E. coli* endotoxin. Results are expressed as % of the amount of ^{51}Cr-endotoxin bound in the absence of drug or unlabelled toxin (100% bound). Concentration of ^{51}Cr-endotoxin was 25 μg ml^{-1}. Data represent the mean with s.d. of 3 separate experiments.

intravenous administration to urethane-anaesthetized guinea-pigs. Three doses of labelled endotoxin were studied, namely 1.0, 3.0 and 6.0 mg kg^{-1}, and experimentally-measured ^{51}Cr-endotoxin binding (in ng mg^{-1} tissue wet weight) for each tissue was standardized by expressing it relative to the administered dose of toxin. The results of these tissue distribution studies are not presented in detail here as they essentially confirmed earlier findings of Mathison *et al.* (1980) showing the liver and spleen as major sites of endotoxin accumulation, with lung, kidney and adrenal gland occupying an intermediate position and skeletal muscle showing very low levels of administered toxin. Since we were primarily interested in endotoxin interactions relevant to *in vivo* toxicity, the accumulation of endotoxin in various tissues was expressed relative to the corresponding level of plasma acid phosphatase activity, which we have previously shown to increase following endotoxin administration, thereby providing a convenient measure of toxicity (Godin & Tuchek, 1983).

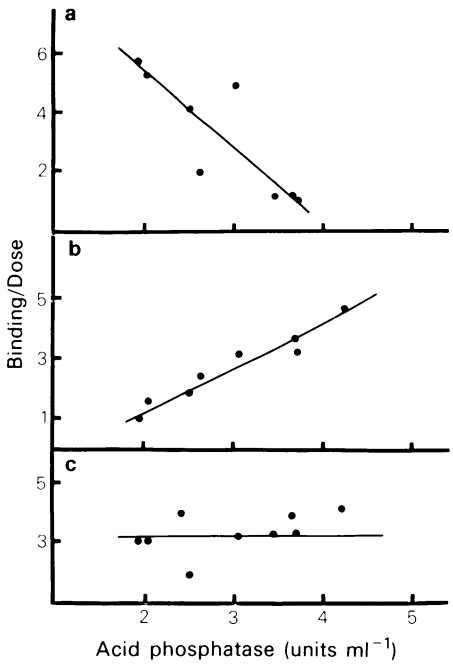

Figure 4 The amount of ^{51}Cr-endotoxin (ng mg^{-1} wet weight) in kidney (a), lung (b) and heart (c) tissues 3 h following the administration of either 1.0 mg kg^{-1} ($n = 3$), 3.0 mg kg^{-1} ($n = 3$) or 6.0 mg kg^{-1} ($n = 2$) ^{51}Cr-*E. coli* endotoxin to guinea-pigs was correlated with plasma acid phosphatase activity. The contribution of dosage to the tissue content of radiolabelled toxin was normalized by dividing the amount of toxin bound in the tissues (ng mg^{-1} wet weight) by the administered dose of toxin in mg kg^{-1}.

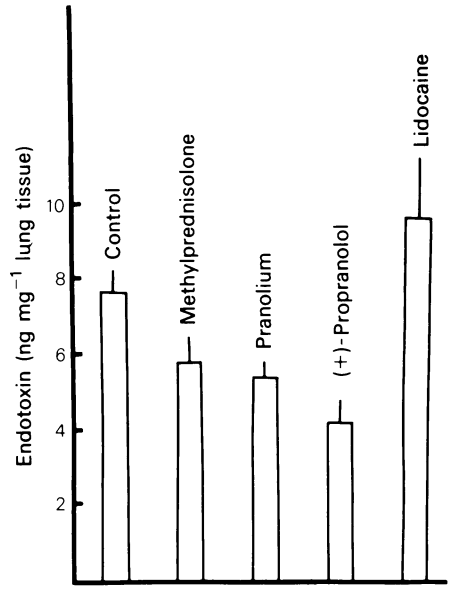

Figure 5 Effect of drug pretreatment on accumulation of ^{51}Cr-endotoxin in guinea-pig lung. Methylprednisolone (35 mg kg^{-1}), pranolium (0.1 mg kg^{-1}) and (+)-propranolol (0.1 mg kg^{-1}) were given as a single intravenous injection to urethane-anaesthetized guinea-pigs 10 min before ^{51}Cr-*E. coli* endotoxin (3.0 mg kg^{-1}) was administered. Lidocaine (1 mg kg^{-1} h^{-1}) was given as a continuous intravenous infusion. Data were obtained at 3 h following endotoxin injection; vertical lines show s.e.means. In all cases, $n = 5$ except (+)-propranolol ($n = 7$) and controls ($n = 11$). Pranolium ($0.05 > P > 0.025$) and (+)-propranolol ($0.005 > P > 0.002$) were significantly different from controls.

Of the various tissues examined, only lung binding of endotoxin correlated positively ($r = +0.964$) with increasing plasma acid phosphatase activity (Figure 4). Kidney and liver (the latter not shown) exhibited negative correlations while the heart and spleen (the latter not shown) showed no obvious correlation between tissue accumulation and elevation in acid phosphatase activity.

The positive correlation obtained with lung endotoxin binding (Figure 4) and the known susceptibility of the lung to pathological involvement in patients with gram-negative septicaemia (Kaplan *et al.*, 1979) led us to focus on this tissue in our investigation of possible *in vivo* endotoxin antagonism by membrane-active agents administered immediately prior to ^{51}Cr-labelled endotoxin (Figure 5). Propranolol (given as the (+)-isomer, 0.1 mg kg^{-1}) proved the most effective agent in decreasing the accumulation of labelled endotoxin in the lung ($0.002 < P < 0.005$). Pranolium at the same dose

also significantly ($0.02 < P < 0.05$) reduced binding. Lidocaine was ineffective and the reduction in toxin accumulation produced by methylprednisolone ($35\ mg\ kg^{-1}$) did not achieve statistical significance. The accumulation of ^{51}Cr-labelled endotoxin in kidney, liver, heart and spleen was not detectably altered by propranolol administration (data not shown).

Discussion

A major factor determining the high mortality rate of patients in gram-negative septic shock despite aggressive antimicrobial therapy may relate to the unopposed actions of endotoxins released from the cell walls of gram-negative bacteria. Efforts have therefore been focussed on attempts to devise therapeutic interventions which would effectively antagonize the detrimental effects of these macromolecular lipopolysaccharide complexes. Since the overall response to endotoxin *in vivo* undoubtedly involves a multiplicity of mediators and sites of action, attempts to modify endotoxin toxicity by antagonizing only one or a few of its many actions, e.g., those involving prostaglandins (Parratt & Sturgess, 1976; Smith *et al.*, 1982), are unlikely to be successful. Rather, interventions focussing directly on the endotoxin molecule (Hughes *et al.*, 1981; Ziegler *et al.*, 1982) or at a more fundamental level by modifying its interaction with cellular components (Bannatyne *et al.*, 1977) would seem to hold more promise. The potential value of this latter type of approach is illustrated by recent studies from our laboratory which have provided evidence that amphipathic membrane-active drugs are able to offer protection against elevations in plasma acid phosphatase activity, a convenient index of toxicity, and mortality in animals given purified *E. coli* endotoxin (Godin & Tuchek, 1983).

The interaction of *E. coli* endotoxin with the plasma membranes of a variety of cell types (Davies *et al.*, 1978) has been shown to be associated with alterations in their functional integrity which, in some cases, may be relevant to the many and diverse consequences of endotoxin administration *in vivo*. The binding of endotoxin to platelets causes a dose-dependent release of 5-hydroxtryptamine and adenine nucleotides which may be important in determining the vascular and haemostatic disorders observed in gram-negative sepsis (Hawiger *et al.*, 1977) and these may be important in the pathogenesis of endotoxic shock (Caridis *et al.*, 1972). Finally, endotoxin can cause the release of lysosomal enzymes from the lung: these may originate either from sequestered leucocytes or possibly from the lung tissue itself (Demling *et al.*, 1980).

The present studies using the erythrocyte as a model membrane system have demonstrated that membrane-endotoxin interaction can be effectively reduced by certain amphipathic drugs such as propranolol and pranolium, which approach the efficacy of unlabelled endotoxin in antagonizing the binding of ^{51}Cr-labelled toxin to the erythrocyte membrane (Figure 2). Previous studies in our laboratory using phospholipase A-modified erythrocytes have implicated phospholipids in membrane–endotoxin interaction (Godin *et al.*, 1982), suggesting that lipophilicity may be an important factor influencing the effectiveness of drugs as antagonists of endotoxin binding. However, the relative inactivity of methylprednisolone (Figure 2) and lidocaine (Figure 3) in this regard suggests a degree of structural selectivity is also involved in determining the ability of amphipathic substances to act as endotoxin antagonists. The superior effectiveness of propranolol as an endotoxin antagonist as revealed in these *in vitro* binding studies parallels the greater protective actions of (+)-propranolol (an isomer retaining the membrane actions and related inhibition of endotoxin binding of the racemate but which is relatively devoid of β-adrenoceptor blocking properties) relative to other membrane-active agents in rats or mice challenged with *E. coli* endotoxin *in vivo* (Godin & Tuchek, 1983). The greatest reduction in the accumulation of ^{51}Cr-labelled *E. coli* endotoxin in lung following its administration to guinea-pigs (Figure 5) was also found with the (+)-isomer of propranolol (the racemate not being well tolerated by the endotoxin-treated animals). The positive correlation (Figure 4) between endotoxin accumulation in the lung and plasma acid phosphatase activity, a useful index of *in vivo* endotoxin toxicity (Godin & Tuchek, 1983), and the well-known involvement of the lung in the pathological manifestations of endotoxaemia (Robin *et al.*, 1972; Kaplan *et al.*, 1979; Krausz *et al.*, 1981) suggest some relationship exists between the protective effects of (+)-propranolol noted previously (Godin & Tuchek, 1983) and the reduction in toxin accumulation by lung tissue. Platelets and granulocytes, both of which are capable of binding endotoxin (Hawiger *et al.*, 1977: Aasen & Ohlsson, 1978), have been shown to become entrapped by the lung in animals and humans with septic shock. However, the poor correlation of these events with alterations in respiratory function (Krausz *et al.*, 1981) suggests that direct interaction of endotoxin with pulmonary endothelium may be largely responsible for the observed pathological changes. Inference by (+)-propranolol with such an interaction at the cellular level, therefore, may be one important component of its protective effects. Another component of the pulmonary protective effect offered by (+)-propranolol that is probably also important in determining the tissue selectivity observed is the preferen-

tial accumulation of propranolol in lung tissue following its administration *in vivo* (Black *et al.*, 1965; Hayes & Cooper, 1971; Schneck *et al.*, 1977). Thus, the antagonistic action of propranolol to endotoxin binding at the membrane level coupled with its tissue distribution characteristics give it the qualities of being a potentially useful endotoxin antagonist *in vivo*.

In contrast to the direct correlation between ^{51}Cr-endotoxin accumulation in lung with toxicity as reflected in plasma acid phosphatase measurements, some tissues such as heart and spleen do not show any such correlation (Figure 4, data not shown for spleen). In other organs such as kidney and liver, ^{51}Cr-endotoxin accumulation correlates negatively with toxicity (Figure 4, data not shown for liver). This negative correlation may be a consequence of toxin-related decreases in tissue perfusion which limit its accumulation in those tissues. In view of these complexities we chose not to examine the effects of drug pretreatment on endotoxin binding to tissues other than the lung. However, it seems likely, on the basis of our model studies using red cells, that the antagonism of endotoxin-tissue interactions by (+)-propranolol may occur in tissues other than the lung and that this general property is largely responsible for the ability of (+)-propranolol to offer protection against the effects of *E. coli* endotoxin *in vivo* (Godin & Tuchek, 1983).

The usefulness of erythrocytes as a model membrane system in mechanistic studies of bacterial toxin action has also been revealed by other investigations. Thus, a direct relation between the susceptibility of different strains of mice to the lethal effects of *Salmonella* endotoxin and the affinity of red cells for the endotoxin has been reported (Hill & Weiss, 1964). It has also been found that a parallelism exists between the protective effects of phenothiazines against the lethal effects of streptolysin O, an exotoxin produced by haemolytic streptococci, and the ability of these drugs to prevent toxin-induced haemolysis of erythrocytes *in vitro* (Besluau *et al.*, 1979). Finally, the ability of staphylococcal α-toxin to bind to and haemolyze erythrocytes from various animal species sensitive to its lethal effects has implicated cellular membranes in its as yet unexplained mechanism of action (Harshman, 1979). Red cell membranes, therefore, should continue to provide a readily available and valuable model system in exploring molecular aspects of bacterial toxin-cell interaction and in the screening of various pharmacological agents as potential antagonists of their action *in vivo*.

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