Effects of Lipid A and Liposomes Containing Lipid A on Platelet and Fibrinogen Production in Rabbits

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The effect of the lipid A moiety of endotoxin on platelet and fibrinogen production was studied in rabbits. Lipid A was infused intravenously in doses ranging from 1 to 100 µg/kg body mass; 18 hr later, selenomethionine-35Se was injected intravenously and its incorporation into fibrinogen and platelets determined. Lipid A in saline stimulated fibrinogen and platelet production, but the dose required was 50–100 times that required for an intact endotoxin. Although lipid A solubilized in triethylamine (TEA) was at least 80 times more active in the Limulus amebocyte lysate assay than was lipid A suspended in saline, the sensitivity of platelet and fibrinogen production to solubilized lipid A was increased only twofold.

Incorporation of lipid A into liposomes had no effect on its Limulus activity. Lipid A in liposomes continued to stimulate platelet, but not fibrinogen, production. Leukopenia that was induced by lipid A in TEA did not occur when rabbits received the same dose of lipid A in liposomes. Lipid A, like intact endotoxin, can stimulate platelet and fibrinogen production and induce leukopenia but the doses required are high. The low solubility of lipid A in aqueous solutions may be only one factor that determines its biologic activity.

ENDOTOXIN (lipopolysaccharide or LPS) from gram-negative bacterial cells consists of a polysaccharide with repeating units (0 antigen), an oligosaccharide (core antigen), and a glycolipid (lipid A). The components of lipid A are similar or identical in most species of Enterobacteriaceae. Lipo- polysaccharides have numerous biologic activities, many of which are apparently caused by the lipid A moiety. In the bacterial cell, the lipid A of LPS is imbedded in a matrix consisting of phospholipid and protein. Lipid A from LPS can be incorporated into liposomes (artificial phospholipid bilayer membranes) in vitro, and in this form it is immunogenic, binds antibodies, and retains adjuvant properties, but lacks the ability to fix complement. Lipopolysaccharides have profound effects on the coagulation system in animals. In a previous investigation we developed a rabbit model in which fibrinogen and platelet production could be quantitated simultaneously after endotoxin infusion; production of these blood components was stimulated by E. coli endotoxin in doses as low as 0.5 µg/kg. In preliminary experiments we found that endotoxin from Shigella flexneri was similar to that from E. coli in its ability to stimulate platelet and fibrinogen production. The role of lipid A in stimulation induced by endotoxin has not previously been demonstrated.

In the present study we investigated the effects of lipid A (prepared from Shigella flexneri) and liposomes containing lipid A on platelet and fibrinogen production in rabbits. We also compared the activity of lipid A observed in the rabbit model with the activity of lipid A in the Limulus amebocyte lysate assay.

MATERIALS AND METHODS

Male New Zealand white rabbits (obtained from the Research Animal Section, Center for Disease Control, Atlanta) were used for all experiments. Their average body mass was 1.98 kg. The animals were received at least 3 days before use, housed in air-conditioned rooms, and given food and water ad libitum.

Lipid A was prepared by acetic acid hydrolysis of Shigella flexneri LPS (isolated by the Westphal method, lot No. 509633, Difco, Detroit, Mich.), and incorporated into liposomes as described. In brief, a solution of 1 g of LPS in 100 ml of 1% acetic acid was heated at 100°C for 2 hr. The precipitate (lipid A) that formed after the hydrolysis of LPS was washed three times with water and then lyophilized. The preparation contained 0.4 µmole of phosphatidylcholine/µg lipid A. The liposomes consisted of dimyristoyl phosphatidylcholine, cholesterol, and dicetyl phosphate in molar ratios of 1:0.75:0.1. The lipid A (20 µg/µmole of phosphatidylcholine) was dried in the preparation flask before adding the other liposomal lipid constituents in CHCl3. All of the lipids were then dried together in a rotary evaporator and swollen in 0.15 M NaCl.

The final aqueous suspension contained phosphatidylcholine and lipid A at concentrations of 10 mM and 200 µg/ml, respectively. After swelling, the liposomes were sonicated to clarity (1 hr at 0°C with a Branson Sonifier 185). During sonication, the liposomes were constantly under a stream of nitrogen. After sonication, the suspension was centrifuged at 27,000 g for 10 min, and the clear supernatant was used for all experiments.

Lipid A was prepared in one of three different ways for injection into rabbits. Lipid A was suspended at an initial concentration of 1 mg/ml of sterile water, dissolved at a concentration of 1 mg/ml of 0.5% triethylamine (TEA), or incorporated into liposomes at the concentration indicated above. Each of these preparations was then diluted with 0.9% NaCl to a final volume of 10 ml for infusion into rabbits in doses ranging from 1 to 100 µg/kg. Lipid A that was not in 0.5% TEA was sonicated for 1 min before dilution with 0.9% NaCl.

The preparations were given by means of an infusion pump at a...
constant rate during a 1-hr period through a needle inserted into the right marginal ear vein. Eighteen hours after the completion of the infusion, each rabbit received an intravenous injection of selenomethionine-\(^{75}\)SeM (\(^{75}\)SeM, 15 \(\mu\)Ci, specific activity 255–518 Ci/g, Sethos- tope, E.R. Squibb and Sons, New Brunswick, N.J.). Twenty-four hours after \(^{75}\)SeM injection, the percent incorporation of the radionuclide into fibrinogen and platelets was determined as described previously. Total white blood cell counts were determined with a Coulter Counter.

Lipid A was compared with endotoxin in a Limulus amebocyte lysate assay modified from the method of Levin and Bang. The lysate was purchased from Pyrotell Associates of Cape Cod, Inc., Woods Hole, Mass. The standard endotoxin or the test sample (0.1 ml) was mixed with 0.1 ml of lysate and incubated 1 hr undisturbed at 37°C. The formation of a gel that remained firm when the test tube was inverted was taken as a positive indication of endotoxin. The assay was capable of detecting \(E.\) coli 0113 endotoxin at a concentration of 0.125 ng/ml. The assay was capable of detecting \(E.\) coli 0113 endotoxin at a concentration of 0.125 ng/ml.

RESULTS

The infusion of lipid A stimulated both fibrinogen and platelet production in rabbits. The lowest dose of lipid A (suspended in saline) that stimulated fibrinogen production was 50 \(\mu\)g/kg (Fig. 1). In animals that received this dose, the incorporation of \(^{75}\)SeM into fibrinogen was 1.30% ± 0.18% (SEM), compared to control values of 0.72% ± 0.04% \((p < 0.005)\). Lipid A that had been solubilized in 0.5% TEA stimulated fibrinogen synthesis when infused at a dose of 25 \(\mu\)g/kg \((p < 0.01)\).

Platelet production appeared to be somewhat more sensitive to lipid A than did fibrinogen production (Fig. 2). That is, the lowest dose of lipid A (suspended in saline) that stimulated incorporation of \(^{75}\)SeM into platelets was 25 \(\mu\)g/kg. When solubilized in TEA, lipid A stimulated platelet production at a dose of 10 \(\mu\)g/kg \((p < 0.005)\). The percent incorporation of \(^{75}\)SeM into platelets and fibrinogen in rabbits that received TEA was not different from that in rabbits that received only normal saline.

When liposomes containing lipid A were infused into rabbits, fibrinogen synthesis was not stimulated at any dose tested (i.e., 1, 10, and 50 \(\mu\)g/kg) (Fig. 1). In contrast, liposomes containing lipid A stimulated platelet production above that of saline controls at lipid A doses of 10 and 50 \(\mu\)g/kg \((p < 0.05)\) (Fig. 2). Infusion of liposomes lacking lipid A had no effect on fibrinogen or platelet production.

The infusion of endotoxin into rabbits characteristi- cally induces leukopenia, with the nadir reached within several hours after infusion. Platelet counts decline more slowly and are still decreased 24 hr after infusion. To determine if a similar pattern could be found with lipid A, we infused rabbits with lipid A (10 \(\mu\)g/kg, solubilized in TEA) and measured the levels of white blood cells and platelets for 48 hr. The white blood cells declined after lipid A infusion, reaching their lowest level (1.26 ± 0.28 \(\times\) 10\(^3\)/liter) 2 hr after initiation of the infusion (Fig. 3). The same dose of lipid A in liposomes had no effect on the white blood cell count (Fig. 3). The platelet counts were unaltered by the infusion either of lipid A (10 \(\mu\)g/kg) in TEA or of this same dose of lipid A in liposomes.
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![Fig. 3. Effect of infusion of lipid A (10 μg/kg) on leukocyte counts (mean ± SEM) in rabbits. Lipid A that was in 0.5% TEA, or in liposomes, was diluted with 0.9% NaCl and infused during 1 hr into 4 rabbits. Six controls received 0.5% TEA in 0.9% NaCl.](image)

In the Limulus amebocyte lysate assay, the apparent activity of lipid A was increased 60–320-fold after solubilization in TEA (Table 1). Neither liposomes alone nor 0.5% TEA gave significant activity in the Limulus assay. Liposomes did not enhance the Limulus activity of lipid A, and TEA had no effect on the apparent activity of lipid A under these conditions (Table 1).

**DISCUSSION**

Although numerous biologic activities exhibited by endotoxin have been ascribed to the lipid A moiety,1,3 the effect of lipid A on stimulation of platelet and fibrinogen production had not previously been measured. In the present work we have shown that lipid A, when infused into rabbits, stimulates both fibrinogen and platelet production and induces leukopenia. Platelet production was slightly more sensitive to lipid A than was fibrinogen synthesis, in agreement with the relative sensitivities to intact E. coli endotoxin found in our previous experiments.6

Endotoxin produces a dose-dependent decrease in the peripheral platelet count, as well as a stimulation of platelet production.6 The close parallelism between the magnitudes of these two effects initially suggested that induction of thrombocytopenia might be the mechanism by which endotoxin stimulates platelet production.6 However, additional statistical analyses provided no support for such a mechanism and indicated the need for additional study of this relationship. Similarly, the present results suggest that the stimulatory effect of endotoxin does not depend on a reduction in platelet number. That is, doses of lipid A (10 μg/kg in TEA) that stimulated platelet production caused no prior decrease in the platelet count.

This type of differential effect, which appeared in several aspects of the present study, was enhanced by varying the medium in which the lipid A was administered. For example, solubilization of lipid A (in TEA) halved the dose required to stimulate fibrinogen or platelet production, whereas incorporation of lipid A into liposomes abolished the stimulation of fibrinogen synthesis but, if anything, enhanced its effect on platelet production. The leukopenia induced by solubilized lipid A followed the same temporal pattern as that produced by endotoxin6 but did not occur when the same dose of lipid A was given in liposomes (Fig. 3).

Even when solubilized in TEA, the dose of lipid A required for stimulation of platelet or fibrinogen production was considerably greater than the minimum dose of intact E. coli endotoxin (0.5 μg/kg) needed to produce this effect.6 It appeared that this difference could be due to a number of factors. For example, lipid A was obtained from Shigella flexneri endotoxin; however, in preliminary experiments, this endotoxin was as effective as E. coli endotoxin in stimulating platelet and fibrinogen production. Alternatively, lipid A might have been partly degraded during the acid hydrolysis procedure required for its isolation. Furthermore, even if it was administered to rabbits in the best vehicle currently available, the full dose of lipid A might not have been delivered to the critical site(s) for its action. Or, finally, the possibility does exist that lipid A might not account completely for the hematologic activities of endotoxin.

It therefore seemed important to determine whether
lipid A could, under any circumstances, exert the full quantitative effect of endotoxin. For this reason, we investigated the Limulus assay, which has proved so useful for quantitating endotoxin in vitro. In this system, solubilized lipid A appeared to be at least as effective as an equal quantity of intact endotoxin. By comparison, lipid A in saline had less than 2% as much activity. This lowered effectiveness of lipid A in saline was in agreement with its activity in vivo. The fact that a similar correlation was not seen for lipid A solubilized in TEA (i.e., solubilized lipid A was still far less effective than endotoxin in vivo) suggests that lipid A does not remain soluble in the circulation or that, devoid of its carbohydrate carriers, it is rapidly inhibited or catabolized. Thus, the present study demonstrates that lipid A alone has the ability to stimulate fibrinogen and platelet production and induce leukopenia. The activity cannot, however, be related to intact endotoxin in a quantitative fashion. This situation arises, in part, from the poor solubility of lipid A. Knowledge of the pharmacokinetics and binding of lipid A in vivo, compared with those of endotoxin, should help define other factors that determine its biologic activity.

REFERENCES

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