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Pyrogenicity and Immunogenicity of Lipid A Complexed with Bovine Serum Albumin or Human Serum Albumin

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The lipid A component of bacterial lipopolysaccharides (endotoxins), when complexed to bovine serum albumin (BSA) or human serum albumin (HSA), was shown to be a potent pyrogen. Furthermore, rabbits could be protected against endotoxin fever by immunization with both lipid A·BSA and lipid A·HSA complexes. The results presented in this paper show that lipid A is responsible for the pyrogenic activity of endotoxins and their ability to induce pyrogenic immunity.

Endotoxins (lipopolysaccharides) of gram-negative bacteria, when injected into higher animals, cause a variety of pathophysiological effects (8) among which the febrile response in rabbits has been studied most intensively (16). Repeated injections of endotoxins reduce the pyrogenic response, and there is strong evidence suggesting that this acquired resistance results from a classical immune response involving a 19*S* immunoglobulin specific to the toxic (pyrogenic) portion of the lipopolysaccharide molecules (9, 10). Therefore, to describe acquired resistance to pyrogen, the term immunity (instead of tolerance) was proposed (9, 10).

Recently, evidence was presented that the common lipid A component of lipopolysaccharides represents the toxic center of endotoxins (4, 5, 14), as postulated earlier (19, 20). Lipid A, when complexed to a solubilizing protein carrier, exhibited typical endotoxic effects such as Limulus lysate gelation (22), lethal toxicity, anticomplementary activity, bone marrow necrosis, and pyrogenicity (5).

In the present paper, the pyrogenic properties of lipid A complexed to bovine serum albumin (BSA) and human serum albumin (HSA) have been studied in more detail. It will be shown that the lipid A·protein conjugates are potent pyrogens comparable in activity to S- and R-form lipopolysaccharides. Furthermore, both lipid A·BSA and lipid A·HSA complexes readily induced pyrogenic (cross) immunity.

MATERIALS AND METHODS

Bacteria, lipopolysaccharides, and glycolipid. The strains *Salmonella minnesota S* (S form) and *S. minnesota R595* (Re) were obtained from J. Schlosshardt (Zentrallaboratorium für bakterielle Darminfektionen, Potsdam, DDR [12]), and *S. minnesota* R345 (Rb) was from F. Kauffmann (Statens Seruminstitut, Kopenhagen, Denmark [12]). Bacteria were cultivated (15) and extracted with phenol-water (S form) (21) or phenol-chloroform-petroleum ether (R forms) (3).

Lipid A and complexes of lipid A with BSA or HSA. Lipid A was prepared from the *S. minnesota* R595 glycolipid and the *S. minnesota* R345 lipopolysaccharide by acetic acid hydrolysis (0.1 N, 2.5 h, 100 C) as described previously (4). No 2-keto-3-deoxyoctonic acid could be detected in lipid A preparations (17), indicating that the lipid A preparations were free of bound core-oligosaccharides. Lipid A was complexed with an equal amount (wt/wt) of either crystallized BSA (Calbiochem, lot 62204) or HSA (generously provided by R. Pennell, Blood Characterization and Preservation Laboratories of Protein Foundation, Inc., Jamaica Plain, Mass.). In addition, lipid A was complexed to rabbit serum albumin (RSA; Pentex lot 14). The complexing procedure of lipid A with albumins has been described previously (4, 5). Briefly, lipid A (10 mg) in water (5 ml) was solubilized by addition of triethylamine (5 μliters). This solution was mixed with a solution of either BSA, HSA, or RSA (10 mg) in water (5 ml). The resulting mixture was dried in a rotary evaporator under reduced pressure. The lipid A·albumin complex was redissolved in water and lyophilized. BSA, HSA, and RSA in the concentrations tested (100 μg/kg) were not pyrogenic. Lyo-

philized lipid A·albumin complexes were dissolved in distilled water by gentle warming (37–50 C) and sonic treatment (Mettler Ultrasonic Cleaner, model ME 2.1). This stock solution was stored at 4 C for not longer than 3 weeks. Small amounts of precipitate formed during storage were redissolved by sonic oscillation. Appropriate amounts of the stock solution were diluted with phosphate-buffered saline (pH 7, 0.15 M) prior to the experiment. All buffers, distilled water, glassware, and other equipment used in this study were sterile and pyrogen free.

Animals. Female and male American Dutch rabbits (1.0–1.3 kg) were used under the same conditions previously described (18).

Pyrogenicity assay. The determination of febrile response has been previously described in detail (18). The minimal pyrogenic dose of the lipid A·albumin complexes causing a 1 F rise of temperature 3 h after injection (MPD-3) was estimated, using three concentrations of the complexes and five rabbits for each concentration. The MPD-3 of the lipid A·albumin complexes was calculated on the basis of their lipid A content.

Immunization procedure. Rabbits (5–10 animals) were rendered immune to pyrogens by daily injections (i.v.) of stepwise increasing toxin doses as described earlier (18). Immunization with BSA was carried out similarly; rabbits were injected (i.v.) with 0.7 μg/kg (day 0), 1.4 μg/kg (day 1 and 2), 2.8 μg/kg (day 3 and 4), and 4.2 μg/kg (day 5 and 6). Immunized animals were tested for pyrogenic immunity by challenge with a 100-MPD-3 dose of the homologous toxin 2 days after the last injection (day 8). Immune animals responded to this challenge with a monophasic fever curve.

RESULTS

Determination of pyrogenicity (MPD-3) of lipid A·albumin complexes. The determination of the MPD-3 of a lipid A·BSA complex (lipid A derived from *S. minnesota* R595 glycolipid) is illustrated in Fig. 1 and 2. Three concentrations of lipid A·BSA complex (0.02, 0.2, and 2 μg/kg) were tested in three groups of five rabbits. The corresponding febrile response curves are shown in Fig. 1. The mean temperatures 3 h after injection are plotted against the log of the concentration of lipid A (Fig. 2). The linear regression line is drawn, and the quantity of toxin causing a rise in temperature of 1 F represents the MPD-3 (0.007 μg/kg). The MPD-3 values of the preparations used in this study are summarized in Table 1. Lipopolysaccharides (glycolipid) and lipid A·albumin complexes exhibited similar pyrogenic activity; the MPD-3 values were found to be between 0.0007 and 0.007 μg/kg. Lipid A when complexed to

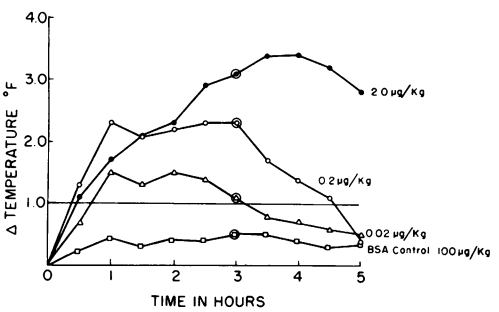

FIG. 1. *Titration of pyrogenic activity of S. minnesota R595 lipid A·BSA complex. Each curve represents the mean febrile response of 5 rabbits injected i.v. with concentrations of lipid A·BSA indicated.*

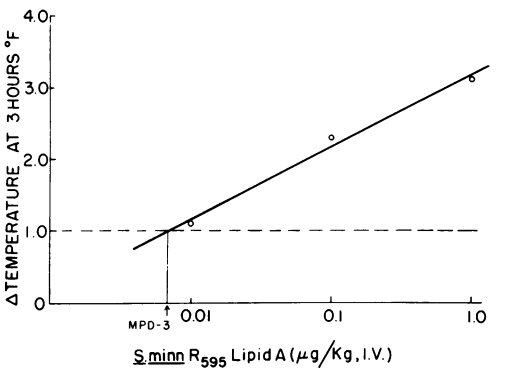

FIG. 2. *Determination of minimal pyrogenic dose (3 h) (MPD-3) of S. minnesota R595 lipid A·BSA complex. Each point represents the mean febrile response of five rabbits 3 h after i.v. injection of the lipid A·BSA complex with indicated concentrations of lipid A. The linear regression line is drawn to 1.0 F and the quantity of toxin at the intercept represents the MPD-3.*

TABLE 1. *Pyrogenic activity (MPD-3) of lipid A·albumin complexes as compared with the parent lipopolysaccharides (glycolipid)*[a]

Organism	Lipopolysaccharides (glycolipid) and lipid A·albumin complexes	Pyrogenicity (MPD-3)
Salmonella minnesota R595	LPS[b]	0.005[c]
	Lipid A·BSA	0.007
S. minnesota R345	LPS	0.0007
	Lipid A·BSA	0.001
	Lipid A·HSA	0.010
	Lipid A·RSA	0.005
S. minnesota S	LPS	0.005
Albumin controls	BSA	>100
	HSA	>100
	RSA	>100

[a] The MPD-3 of lipid A·albumin complexes is calculated on the basis of their lipid A content.
[b] LPS, Lipopolysaccharide.
[c] Measured as micrograms per kilogram.

HSA, the latter being less soluble than BSA and RSA, had a MPD-3 value of 0.01 μg/kg.

Reciprocal cross-immunity tests between lipid A complexed to BSA and S. minnesota S lipopolysaccharide. Rabbits were immunized with lipid A·BSA (lipid A from *S. minnesota* R595 glycolipid) and tested for immunity with a 100 MPD-3 dose of the complex (0.7 μg/kg). Immunity to lipid A·BSA was achieved (Fig. 3). Two days later (day 10) the immune animals were tested with BSA (0.7 μg/kg), and no significant fever response (Δ temperature = 0.8 F at 3 h) was observed (Fig. 3). To assay for cross-immunity, the lipid A·BSA immune rabbits were challenged 2 days later (day 12) with a 100-MPD-3 dose (0.5 μg/kg) of *S. minnesota S* lipopolysaccharide. Complete cross-immunity was observed (Fig. 3).

To demonstrate reciprocal cross-immunity, rabbits were rendered immune to *S. minnesota S* lipopolysaccharide (Fig. 4, control and immune). On challenge of the immune animals with a 100-MPD-3 dose of the lipid A·BSA complex, complete cross-immunity could be

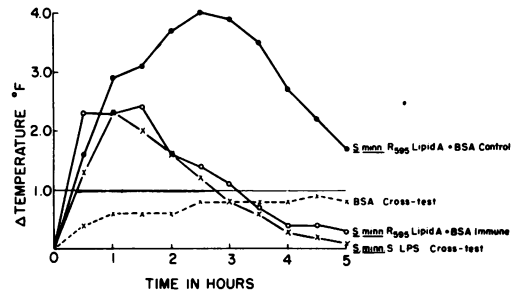

FIG. 3. *Cross-immunity between S. minnesota R595 lipid A·BSA and S. minnesota S lipopolysaccharide. Lipid A·BSA immune animals tested with BSA and S. minnesota S lipopolysaccharide. Each curve represents the mean febrile response of five rabbits injected i.v. with a 100-MPD-3 dose of toxin.*

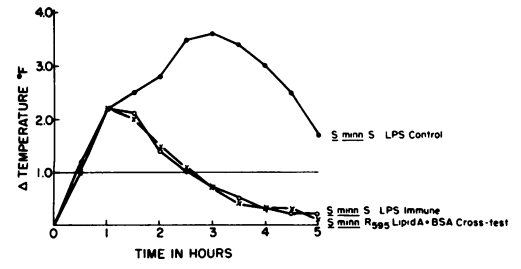

FIG. 4. *Reciprocal cross-immunity between S. minnesota S lipopolysaccharide and S. minnesota R595 lipid A·BSA. S. minnesota S lipopolysaccharide immune animals tested with lipid A·BSA. Each curve represents the mean febrile response of five rabbits injected i.v. with a 100-MPD-3 dose of toxin.*

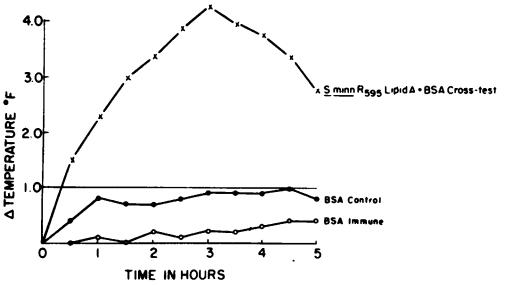

FIG. 5. *Non-cross-immunity test between BSA and S. minnesota R595 lipid A·BSA. BSA-immunized animals tested with lipid A·BSA. Each curve represents the mean febrile response of five rabbits.*

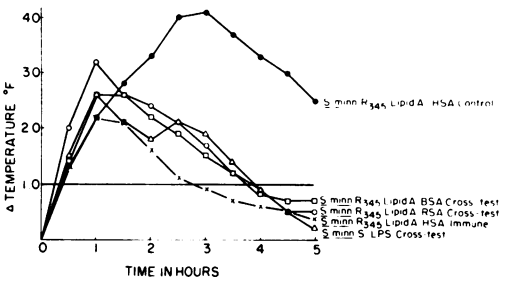

FIG. 6. *Cross-immunity between S. minnesota R345 lipid A·HSA and S. minnesota R345 lipid A·BSA and S. minnesota S lipopolysaccharide, respectively. In addition, the lipid A·HSA immune animals were cross-tested with S. minnesota R345 lipid A·RSA. Each curve represents the mean febrile response of five rabbits injected i.v. with a 100-MPD-3 dose of toxin.*

demonstrated (Fig. 4). In a control experiment, rabbits were immunized with BSA only (see Materials and Methods). When these animals were injected with BSA (0.7 μg/kg) 2 days later (day 10), no rise of the body temperature was observed. A normal biphasic fever curve was obtained however when the BSA-immunized rabbits were challenged 2 days later (day 12) with a 100-MPD-3 dose of lipid A·BSA (Fig. 5).

Cross-immunity test between lipid A complexed to HSA and lipid A·BSA and S. minnesota S lipopolysaccharide. Rabbits immunized with lipid A·HSA complex (lipid A from *S. minnesota* R345 lipopolysaccharide) showed good immunity when challenged with a 100-MPD-3 dose (1 μg/kg) of the homologous complex on day 8 (Fig. 6). The lipid A·HSA immune rabbits were then cross-tested with 100-MPD-3 doses of lipid A·BSA (0.1 μg/kg, day 10) and *S. minnesota S* lipopolysaccharide (0.5 μg/kg, day 12). Complete cross-immunity to all toxins tested could be demonstrated (Fig. 6). In addition, on day 14 the immune animals were cross-tested with a 100-MPD-3 dose of lipid A

complexed to RSA (0.5 μg/kg). Good cross-immunity is evident (Fig. 6).

DISCUSSION

It is well known that endotoxins (lipopolysaccharides) of *Salmonella* wild-type (S) strains are potent pyrogens (16, 18, 19). The demonstration that lipopolysaccharides (glycolipids) derived from R-mutant strains exhibit similar pyrogenicity (11, 12) indicated that the polysaccharide portion of lipopolysaccharides does not contribute specifically to pyrogenicity, but rather that the common lipid A component represents the pyrogenic center of endotoxins. Recently, it could be shown that purified lipid A, when complexed to solubilizing carriers, exhibited endotoxic activity in a number of endotoxin tests, including fever induction in rabbits (5). In the experiments presented in this study, lipid A complexed to BSA or HSA was used. Both lipid A·BSA and lipid A·HSA complexes exhibited pyrogenicity comparable to that of S- and R-form lipopolysaccharides. Thus, the pyrogenic principle of endotoxins is clearly embedded within their lipid A component.

Since the classical work of Beeson (1, 2), it has been well known that repeated injections of endotoxin into rabbits will render these animals resistant to pyrogenic (and lethal) activity of endotoxin, a phenomenon often referred to as "pyrogenic tolerance" (1, 7, 18). It was also shown that pyrogenic cross-tolerance could be induced by serologically nonrelated lipopolysaccharides (10, 18) as well as by R-mutant endotoxins (11). Furthermore, pyrogenic tolerance could be passively transferred by serum (9, 10). From these observations it was concluded that acquired endotoxin resistance resulted from a classical immune mechanism involving 19S antibodies specific for lipid A but distinct from O- or R-specific immunoglobulins. Consequently, it was proposed to substitute the term immunity for the term tolerance (9, 10).

If pyrogenic immunity is a result of induction of lipid A-specific antibodies, it should be possible to protect animals against endotoxin fever by immunization with lipid A preparations. In earlier attempts, lipid A prepared from S-form lipopolysaccharides was shown to be a poor immunogen (18). In the present study, lipid A complexed to solubilizing protein carriers has been employed for immunization. It was shown that lipid A·BSA complexes readily induce immunity to homologous pyrogens as well as cross-immunity to heterologous pyrogens. A control experiment demonstrated that BSA itself did not participate specifically in lipid A·BSA-induced immunity, since a 100-MPD-3 dose of lipid A·BSA elicited in BSA-immunized rabbits a normal fever response. In animals immunized with *S. minnesota S* lipopolysaccharide, reciprocal cross-immunity to lipid A·BSA could be demonstrated. Cross-immunity to various lipid A·protein complexes and *S. minnesota S* lipopolysaccharides could also be achieved by immunizing rabbits with a lipid A·HSA complex.

Free lipid A, when solubilized with pyridine (18) or triethylamine (E. T. Rietschel, unpublished data), is pyrogenic but to a much lower degree (MPD-3 = 0.2 μg/kg). However, when complexed with BSA, RSA, and HSA, lipid A exhibits pyrogenic activity comparable to that of S- and R-form lipopolysaccharides. It appears therefore, that carriers such as albumins, by providing solubility and possibly exposing toxophore groups or a toxic conformation (13) within lipid A, allow the expression of optimal endotoxic activity of lipid A as previously suggested (4, 5). The results presented and discussed in this paper suggest, in addition, that carriers such as BSA and HSA play a significant role for the immunogenic properties of lipid A. It is probable that O- and R-poly(oligo)saccharides of endotoxins play a comparable role as carriers of lipid A.

Milner and Rudbach (Bacteriol. Proc. 96, 1968) and Griesman et al. (6, 7) using S-form lipopolysaccharides have demonstrated that pyrogenic tolerance is a biphasic phenomenon which can be separated into an early and a late phase. The early phase of tolerance exhibits no interendotoxin specificity and is thought to be due to cellular factors. The late phase of tolerance appears to be specific and to be mediated mainly by O-specific immunoglobulins. However, antibodies with broader specificity were also shown to participate in the late phase tolerance (6, 7). In the present paper, lipid A preparations, free of O-polysaccharides, have been investigated. Preliminary experiments show that lipid A·albumin complexes readily induce the early phase of tolerance. However, this early tolerance is transient as demonstrated by the febrile response to daily injections of lipid A·albumin complexes. Therefore, the lipid A-induced pyrogenic tolerance (immunity) described in this paper appears to be distinct from the early phase of tolerance.

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