Anticomplementary Activity of Lipid A Isolated from Lipopolysaccharides[1] (37462)

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The isolation and characterization of the lipopolysaccharides (LPS) of R mutant strains of Enterobacteriase (particularly the mutants of Salmonella sp whose LPS lacks heptose) has provided evidence that lipid A is responsible for the endotoxic property of LPS (1). In addition, recent experiments have shown that isolated lipid A preparations under suitable conditions can cause pyrogenicity in rabbits and also inactivate serum complement (2, 3).

Lipid A, as prepared by mild acid hydrolysis of LPS, is insoluble in water, but may be rendered soluble by the addition of triethylamine (TEA) to a final concentration of 0.01–1% (1). Galanos *et al.* (2), however, reported that neither the insoluble lipid A nor lipid A solubilized with TEA possessed any anti-complementary activity. They were able to restore anticomplementary activity only after addition of a carrier molecule, such as bovine serum albumin (BSA), followed by removal of TEA by flash evaporation. They thus proposed the formation of a soluble complex of BSA–lipid A. This report is concerned with the physical relationship of BSA and lipid A, and in particular, whether or not lipid A alone is sufficient to produce the observed biological effects. This study appears in order since mixtures of BSA–lipid A have recently been studied in a number of other biological systems (4, 5).

We first attempted to use this proposed BSA–lipid A complex for *in vivo* depression of complement levels in rabbits. Because of the capability of using homologous serum albumin to form the postulated complex, it was our hope that decomplementation could be obtained by mechanisms more nearly physiological than those previously described (6). These initial experiments led us to similar conclusions for the *in vivo* anticomplementary activity of lipid A as that described *in vitro*. However, the lack of any correlation between the serum levels of radiolabeled rabbit serum albumin and lipid A caused us to examine in more detail the properties of this albumin–lipid A mixture.

The results of our experiments suggest the following properties of lipid A. (a) Interaction of TEA-solubilized lipid A with BSA was limited to less than 5% of the total BSA present. (b) Soluble lipid A, which has not formed a complex with any carrier molecule retains its anticomplementary property and lethality for mice. (c) The anticomplementary activity of lipid A is highly dependent upon its degree of solubility.

Materials and Methods. Escherichia coli 0111:B4 (ATCC 12015) was used in all experiments. Bacteria were routinely grown in a fermenter at 37° with aeration in a minimal salts medium (7). Tritium-labeled cells were obtained by growth of the bacteria in 2 liters of glucose-minimal media described by Ryan (8) supplemented with 5.0 mCi of labeled D-[1-^{3}H] glucose (4.5 Ci/mmole, ICN; Irvine, Calif.). ^{32}P-labeled cells were obtained by growth in 2 liters of phosphate-minimal medium (8) containing 1.0 mCi of carrier-free $Na^{32}PO_4$·HCl (NEN, Boston, Mass.).

LPS was routinely prepared by phenol-water extraction of cell suspensions as previously described (9, 10), and lipid A obtained by mild acid hydrolysis of chloroform-

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washed LPS solutions[2] (11, 12). The lipid A precipitates were washed three times by centrifugation with pyrogen-free distilled water. In some preparations, a final wash with acetone was included. Lipid A preparations were then resuspended at approximately 0.5 mg/ml in pyrogen-free distilled water by brief low-power sonication (Branson Instrument Corp., Danbury, Conn.; half maximal wattage, 4°, 5 sec) and these insoluble suspensions of lipid A (I-LA) used for all subsequent preparations. For the preparation of radiolabeled lipid A, LPS from unlabeled cells was supplemented, prior to acid hydrolysis, with LPS prepared from bacteria grown in the presence of ^{32}P or D-[1-^{3}H] glucose as described above.

TEA solubilized lipid A (TEA-LA) was prepared by adding 1% TEA (Matheson, Coleman & Bell, Los Angeles, Calif.) to I-LA. TEA-LA was either used as such or further treated in one of two ways to remove TEA. In one of these (BSA-LA) an external protein, BSA, was added to TEA-LA in a weight ratio of 1/1 and the mixture dried in a rotary evaporator under reduced pressure as described by Galanos (2). In experiments where ^{32}P-lipid A or ^{3}H-lipid A was used, either ^{125}I-BSA or ^{131}I-BSA, (Na ^{125}I, carrier free, ICN, Irvine, Calif.; Na ^{131}I, carrier free, Cambridge Nuclear, Boston, Mass.) labeled by the chloramine T procedure (13), was added as the external protein. In a second method, lipid A solubilized by TEA was extensively dialyzed against sterile distilled H_2O (D-LA).

Anticomplementary activity was routinely determined by the inhibition of hemolysis of sensitized erythrocytes as previously described (6). Briefly, 200 μl of normal rabbit serum diluted 1/18 were incubated for 30 min at 37° with preparations of lipid A made up to a volume of 100 μl in veronal buffer. Then 200 μl of sensitized erythrocytes were added and the mixture incbated for 60 min at 37°. Under these conditions control tubes containing veronal buffer gave 80% hemolysis. For all hemolysis determinations control incubations with solvent alone were performed. Lethality in mice was assayed by intraperitoneal injections of 0.5 ml of lipid A preparations into 18–20 g female BALB/c mice which were injected simultaneously with 10 μg actinomycin (14). Deaths were recorded at 48 hr. Concentrations of lipid A for toxicity studies were estimated by assaying for glucosamine by the procedure of Elston and Morgan (15).

Velocity sedimentation was carried out in sucrose using 5–20% linear gradients in phosphate buffered saline (pH 7.4). Samples were centrifuged in the Beckman Model L2-65B ultracentrifuge for 4 hr at 300,000*g* and 4° using the swinging bucket rotor. Fractions were collected from the bottom of the tube and assayed for radioactivity.

Gel filtration chromatography was carried out using either Agarose 1.5 A (Biorad, Los Angeles, Calif.) or Sephadex G-200 (Pharmacia, Rahway, New Jersey). All columns were developed with phosphate buffered saline at 4° using a positive pressure of less than one half the column height. Void volumes were determined by exclusion of blue dextran.

Lipid A phosphate was measured by the procedure of Ames (16).

Results. (1) Examination of the interaction of BSA with lipid A. The presence of complexes of lipid A and BSA in the LA-BSA preparations was tested by velocity sedimentation in sucrose gradients and by gel filtration chromatography. When samples of ^{32}P-lipid A-^{125}I-BSA (BSA-LA) prepared as described by Galanos (2) (see Materials and Methods) were layered onto sucrose gradients better than 95% of the ^{32}P was pelleted whereas less than 3% of the ^{125}I was pelleted. Because of the high sucrose concentrations in the gradient fractions, anticomplementary activity could not be satisfactorily determined. For this reason equivalent samples were subjected to chromatography on Agarose 1.5 A.

The results of one such fractionation are shown in Fig. 1a. The ^{32}P-lipid A eluted with the void volume whereas essentially all of the ^{125}I-BSA was retained. Biological

[2] We have routinely used two hydrolysis conditions for preparation of lipid A: 1% acetic acid for 3 hr at 100° or 0.1 *N* HCl for 30–40 min at 100°. Both of these procedures gave similarly active lipid A preparations.

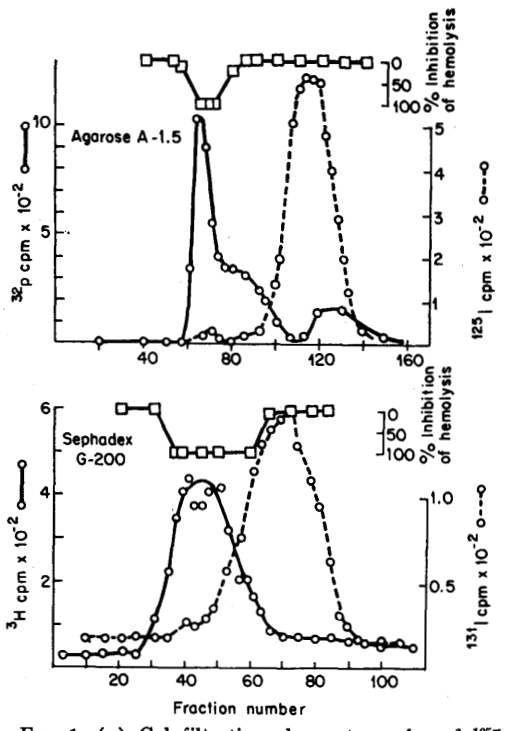

FIG. 1. (a) Gel filtration chromatography of ^{125}I-BSA-LA-^{32}P on Agarose 1.5 A. Approximately 0.5 ml of BSA-LA were applied to a 0.5 cm × 35 cm column. A flow rate of 0.6 ml/hr was maintained and fractions of 0.2 ml were collected. Aliquots of various eluted fractions were counted by liquid scintillation or were assayed for anticomplementary activity. (b) Gel filtration chromatography of ^{131}I-BSA-LA-^{3}H on Sephadex G-200. Conditions were the same as those described above except that fractions of 0.3 ml were collected.

activity, as measured by inhibition of hemolysis was confined to the ^{32}P-lipid A peak.

Very similar results were obtained when preparations of ^{3}H-lipid A were treated with ^{131}I-BSA and then fractionated on Sephadex G-200 (Fig. 1b). Although the resolution of the two peaks is not as complete as with Agarose 1.5 A, the anticomplementary activity is, again, clearly associated with the ^{3}H-lipid A and not with BSA.

The limited extent to which BSA and lipid A may complex suggested that it might be possible to prepare biologically active soluble lipid A in the absence of TEA or carrier molecules.

(2) Preparation of biologically active soluble lipid A. In a series of experiments designed to test the biological activity of lipid A as a function of its solubility, ^{3}H-lipid A was prepared as described in Materials and Methods. After the final resuspension in distilled water, the lipid A suspension was divided into four equal fractions. Three of these fractions were solubilized with TEA and subsequently treated exactly as described in Materials and Methods to obtain lipid A preparations BSA-LA, TEA-LA, and D-LA. The fourth fraction I-LA was untreated.

Table I indicates that, in each of the procedures described above, better than 90% of the total ^{3}H-lipid A counts are recovered. The specific activity of these preparations, as measured by ^{3}H counts per minute per μmole phosphate, is also shown not to vary substantially.

Each of these four fractions was tested for solubility of ^{3}H-lipid A. Aliquots were centrifuged in the cold for 20 min at 30,000*g* and the number of counts remaining in the supernatant determined. The results shown in Table I indicate that all preparations except I-LA were soluble. Removal of TEA by either flash evaporation, in the case of BSA-LA, or by dialysis, as with D-LA,

TABLE I. Properties of ^{3}H-Lipid A Preparations.

Preparation	^{3}H-recovered (%)	cpm × 10^{-5}/ μmole PO_4	^{3}H-soluble (%)	50% Inhibition (nmole PO_4)[a]
I-LA	100	6.3	6	22
BSA-LA	103	5.8	86	1.2
TEA-LA	98	8.1	97	0.2
D-LA	91	7.6	81	0.2

[a] Amount of lipid A required to give 50% inhibition of hemolysis of 1.0 × 10^{8} sensitized erythrocytes by 200 μl of 1/18 dilution of normal rabbit serum.

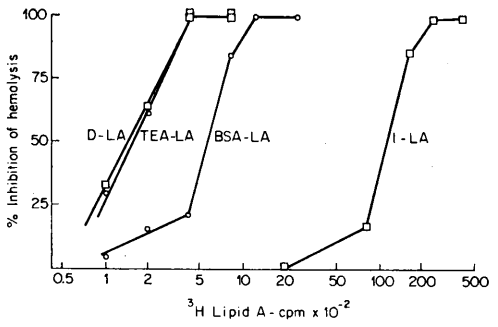

FIG. 2. Inhibition of hemolysis by preparations of lipid A. The determination of percent inhibition of hemolysis by D-LA; TEA-LA; BSA-LA; and I-LA; were as described in the text.

appeared to have only a slight effect on the solubility of the lipid A.

The anticomplementary activity of each of these fractions was then determined by incubation of various dilutions of the lipid A preparations with normal rabbit serum as described earlier. The number of ^{3}H-lipid A counts required to produce an inhibition of hemolysis of 50% was then determined. The results of one such titration are shown in Fig. 2, and the 50% inhibition point normalized to nmole PO_4 indicated in Table I.

It can be seen from Fig. 2 that, although I-LA retains some anticomplementary activity, this activity is enhanced several orders of magnitude after solubilization with TEA. Furthermore, the activity is retained when dialyzable TEA is removed in the absence of carrier protein (D-LA). The procedure used for TEA removal in the presence of BSA appears to have some deleterious effect on the subsequent anticomplementary activity as determined by the increasing amount of lipid A required for 50% inhibition of hemolysis.

A further assay of the biological activity associated with carrier-free preparation of lipid A (D-LA) was lethality to mice. Due to a limited amount of lipid A, the sensitivity of the mice was increased by the simultaneous injection of 10 μg of actinomycin D. The results of this study, Table II, showed that D-LA was toxic to mice, having, under these conditions, an LD_{50} of about 2.0 μg of lipid A.

Discussion. The data reported here confirm the earlier observations of the potent anticomplementary activity of lipid A after treatment with BSA. The interpretation that solubilization is associated with a complexing of lipid A and BSA molecules, while not inconsistent with the earlier observations, is not substantiated in the current experiments. It appears, instead, that the process of solubilization with TEA and subsequent removal of free TEA is, in itself, sufficient to render the lipid A soluble.

The experiments described in this report cannot exclude the possibility of a limited interaction of BSA with lipid A. Based upon the chromatographic fractionation of BSA-LA on Agarose 1.5 A, a maximum of 5% of the BSA is eluted with the anticomplementary ^{32}P-lipid A peak (Fractions 60–80; Fig. 1a). If it is assumed that in these fractions, all the lipid A is coupled to BSA, then one can calculate a ratio of 700 monomer lipid A molecules (aggregate molecular weight 1.5×10^6 daltons) per BSA molecule (molecular weight 7×10^4 daltons). While such an interaction is not impossible, a more probable interpretation of the data would be an aggregation of a small amount of protein during the flash evaporation to dryness during the preparation of BSA-LA, a procedure known to cause aggregation in other protein systems (17).

The concept of a carrier-free soluble lipid A aggregate with biological activity is further supported by experiments where unbound TEA solubilizer was removed by dialysis in the absence of carrier molecules. Although TEA is freely dialyzable, the possibility that a fraction of the TEA is tightly bound to the lipid A, and consequently not removed by dialysis, cannot be excluded by the above experiments. An extension of this hypothesis might suggest that, in fact, TEA complexed

TABLE II. Lethality of D-LA in BALB/c Mice.

Dose	Survivors[a]
24 μg	0/8
16 μg	1/8
8 μg	1/9
4 μg	3/8
2 μg	3/8
1 μg	7/9

[a] Recorded after 48 hr.

to lipid A is necessary for lipid A solubility.

It is more difficult to reconcile the observations of Galanos *et al.* (2) that neither of their preparations of lipid A (I-LA and TEA-LA in our notation) possessed anticomplementary activity. Whereas I-LA appears to be only approximately 1/20th as biologically active as BSA-LA, it still retains the ability to inhibit hemolysis. This is not without precedent as it has been observed earlier by Mergenhagen *et al.* (18) that the LPS from *S. minnesota* Re595 (containing only KDO and lipid A) is highly anticomplementary. As those studies were done in the absence of solubilizer or carrier, it is presumed that the preparations of LPS from Re595 were equally as insoluble as I-LA. The reasons for the lack of anticomplementary activity in Galanos' preparation of TEA-LA are unclear although it should be pointed out that those experiments were done using guinea pig serum while those of the present study used rabbit serum.

One important aspect of these experiments is the strong dependence of biological activity upon the physical state of the lipid A. This was observed in 1969 by Westphal *et al.* (1) who demonstrated an almost tenfold increase in pyrogenicity of Re595 LPS after solubilization in 0.01% TEA. This increase in biological activity has also been observed in the present studies when lipid A was solubilized with TEA. In addition, in preliminary studies, procedures which insolubilized approximately 50% of TEA-LA were accompanied by a tenfold drop in the biological activity as measured by anticomplementary activity.

In addition to the biological activities of the lipid A preparations as determined by toxicity and complement activation, we are presently extending our studies to examine the interaction of lipid A with other biological systems. Such studies include the activation of Hageman factor (Factor XII in the clotting system of plasma) and the interaction of lipid A with individual components of the complement system.

Summary. We have examined in detail the relationship between the solubility of the isolated lipid A portion of bacterial lipopolysaccharides and biological activity. Lipid A isolated by mild acid hydrolysis of the LPS of *E. coli* 0111:B4, was shown to be an inactivator of complement. This anticomplementary activity was increased over two orders of magnitude after solubilization with triethylamine. In contrast to previously published reports, however, highly active soluble lipid A preparations could be obtained independent of a carrier protein. When such a carrier was present, better than 95% of the carrier did not contribute to the formation of a soluble complex. We conclude that the biological activity of lipid A is highly dependent upon its solubility.

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1. Westphal, O., Gmeiner, J., Lüderitz, O., Tanaka, A., and Eichenberger, E., Colloq. Int. Center. Nat. Rech. Sci. **174**, 69 (1969).

2. Galanos, C., Rietschel, E., Lüderitz, O., and Westphal, O., Eur. J. Biochem. **19**, 143 (1971).

3. Galanos, C., Rietschel, E., Lüderitz, O., Westphal, O., Kim, Y. B., and Watson, D. W., Eur. J. Biochem. **31**, 230 (1972).

4. Yoshida, M., Rietschel, E., Galanos, C., Hirata, M., Lüderitz, O., and Westphal, O., Japan. J. Med. Sci. Biol. **24**, in press.

5. Alexander, P., and Evans, R., Nature (London) **232**, 76 (1971).

6. Cochrane, C. G., Müller-Eberhard, H. J., and Aikin, B. S., J. Immunol. **105**, 55 (1970).

7. Vogel, H. J., and Bonner, D. M., J. Biol. Chem. **218**, 97 (1956).

8. Ryan, J. L., PhD thesis, Yale University, New Haven, Conn. (1969).

9. Westphal, O., and Jann, K., *in* "Methods in Carbohydrate Chemistry" (R. L. Whistler, ed.), p. 83. Academic Press, New York (1965).

10. Leive, L., Shovlin, V. L., and Mergenhagen, S. E., J. Biol. Chem. **243**, 6384 (1968).

11. Burton, A. J., and Carter, H. E., Biochemistry **3**, 411 (1964).

12. Morrison, D. C., and Leive, L., Manuscript in preparation. (1973).

13. McConahey, P. J., and Dixon, F. J., Int. Arch. Allergy Appl. Immunol. **29**, 185 (1966).

14. Johnson, K. J., and Ward, P. A., J. Immunol. **106**, 1125 (1971).

15. Elston, L. A., and Morgan, W. T., Biochem. J. 27, 1824 (1933).

16. Ames, B. N., and Dubin, D. T., J. Biol. Chem. 235, 769 (1960).

17. Putnam, F. W., *in* "The Proteins" (H. Neurath and K. Bailey, eds.), p. 807. Academic Press, New York (1953).

18. Mergenhagen, S. S., Gewurz, H., Gladen, H. A., Nowotny, A., Kasai, N., and Lüderitz, O., J. Immunol. 100, 227 (1968).

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