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Endotoxic Activity of Complexes of Myristic Acid and Proteins (39189)

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Endotoxin is a component of the outer cell wall of the enteric bacteria (1). Chemically, endotoxin from wild-type bacteria is a lipopolysaccharide (LPS) composed of two distinct regions: (a) a hydrophilic portion which is a polysaccharide bearing the *O*-specific chains and (b) a hydrophobic lipid portion called lipid A (2). Subsequent investigations established that lipid A consists of glucosaminyl-β-1,6-glucosamine disaccharides whose hydroxyl and amino groups are substituted by long-chain fatty acids (3). Free lipid A can be prepared from LPS by mild acid hydrolysis which cleaves the linkage of the polysaccharide to lipid A. As a result, lipid A is released as a water-insoluble material devoid of biological activity (4). Soluble lipid A complexes can be prepared by binding lipid A to a hydrophilic carrier molecule such as bovine serum albumin (5). Such complexes possess many of the attributes of endotoxin (6), indicating that lipid A is the active center of LPS whereas the polysaccharide portion is the solubilizing carrier. Recently, a glycolipid (*N*-palmitoyl D-glucosamine) having at least one property of endotoxin, that is, mitogenicity for mouse B lymphocytes, has been synthesized (7).

In this study, the capability of lipid–protein complexes to kill mice rendered hyperreactive to endotoxin by mithramycin (8) has been assessed. A serious concern in this investigation has been the possible contamination of the lipid–protein complexes with substances having endotoxic activity. In this report, evidence is presented that myristic acid–protein complexes and dimethyl myristamide–concanavalin A complexes possess endotoxic activity.

Materials and methods. Male BALB/c or ICR mice weighing 22 to 27 g were used in all experiments. Mice were obtained from Flow Research Animals, Inc., Dublin, Virginia. They were allowed to adjust to their new environment for 1 week prior to experimentation. The mice were given free access to water and food (Purina Lab Chow, Purina Ralston Corp., St. Louis, Missouri). All injections were given simultaneously by the intraperitoneal route. Mithramycin (NSC-24559; Charles Pfizer & Co., Clifton, N.J.) and bacterial lipopolysaccharide (Difco Laboratories, Detroit, Mich.) were dissolved or suspended in 0.15 *M* NaCl.

Lipid A was extracted from *Escherichia coli* 0127:B8 lipopolysaccharide (Boivin method; Difco) by a procedure adapted from the method of Galanos *et al.* (4). The absence of 2-keto-3-deoxyoctonate was confirmed by the thiobarbiturate assay (9). Complexes of lipids and bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, Missouri) or concanavalin A (Con A; grade IV Sigma) were prepared according to the methods described by Galanos *et al.* (5). The following lipids were complexed to BSA or Con A: myristic acid, myristoyl choline chloride, myristoleyl alcohol, *N,N*-dimethyl myristamide, DL-12 hydroxystearic acid or β-γ-dimyristoyl-L-α-lecithin (Sigma), β-hydroxymyristic acid (Applied Science Laboratories Inc., State College, Pennsylvania), and acetic acid *N*-hydroxysuccinimide ester, caprylic acid *N*-hydroxysuccinimide ester, lauric acid *N*-hydroxysuccinimide ester, palmitic acid *N*-hydroxysuccinimide ester, or stearic acid *N*-hydroxysuccinimide ester (ICN Pharmaceuticals Inc., Cleveland, Ohio). Immediately before use, the complexes were suspended in distilled water and dispersed with sonication.

Results. The mithramycin-treated mouse was used as an indicator of endotoxin activity by the lipid–protein complexes. Of the 17 complexes tested, only 6 possessed presumptive endotoxic activity (Table I). Of the seven complexes of lipids with BSA, only one complex (myristic acid–BSA) possessed endotoxic activity. Of the 10 complexes of lipids with Con A, five complexes killed some mithramycin-treated mice. Of these five complexes, only myristic acid–

TABLE I. DETECTION OF ENDOTOXIN ACTIVITY IN LIPID–PROTEIN COMPLEXES USING MITHRAMYCIN-TREATED MICE AS THE INDICATOR.[a]

Test material[b]	Dose	Number of mice	Percentage dead
Lipid A-Con A	5 mg/kg	30	90
Myristic acid	50 mg/kg	20	0
Myristic acid-BSA	50 mg/kg	45	62
Myristic acid-Con A	100 mg/kg	25	32
Myristic acid-Con A	50 mg/kg	20	20
Dimethyl myristamide	50 mg/kg	20	0
Dimethyl myristamide-Con A	50 mg/kg	77	49
Dimethyl myristamide-BSA	50 mg/kg	20	0
β-Hydroxymyristic acid	50 mg/kg	20	0
β-Hydroxymyristic acid-Con A	50 mg/kg	50	16
β-Hydroxymyristic acid-Con A	25 mg/kg	30	17
β-Hydroxymyristic acid-BSA	50 mg/kg	20	0
Myristoleyl alcohol-Con A	50 mg/kg	20	30
Myristoleyl alcohol-BSA	50 mg/kg	20	0
Acetic acid *N*-hydroxysuccinimide ester-Con A	50 mg/kg	20	25
Acetic acid *N*-hydroxysuccinimide ester-BSA	50 mg/kg	20	0
Caprylic acid *N*-hydroxysuccinimide ester-Con A	50 mg/kg	20	0
Dimyristoyl lecithin-Con A	50 mg/kg	21	0
Hydroxystearic acid-Con A	50 mg/kg	20	0
Lauric acid *N*-hydroxysuccinimide ester-Con A	50 mg/kg	20	0
Palmitic acid *N*-hydroxysuccinimide ester-BSA	50 mg/kg	20	0
Stearic acid *N*-hydroxysuccinimide ester-Con A	50 mg/kg	20	0
Stearic acid *N*-hydroxysuccinimide ester-BSA	50 mg/kg	20	0
Concanavalin A	50 mg/kg	30	0
Bovine serum albumin	50 mg/kg	20	0
Lipid A-BSA	5 mg/kg	20	60

[a] Male BALB/c mice were injected ip with the indicated dose of the test material and 0.5 mg of mithramycin/kg. None of these agents alone, at the doses indicated, killed any of the treated mice. Lethality was scored on Day 4.

[b] Con A, concanavalin A complex; BSA, bovine serum albumin complex.

Con A and dimethyl myristamide–Con A consistently killed mice. It should be noted that concanavalin A, bovine serum albumin, myristic acid, dimethyl myristamide, or β-hydroxymyristic acid individually did not kill mice.

Mice treated with lipid A–Con A complex developed resistance to a dose of LPS that killed untreated mice (8). Mice were treated with 12 lipid–protein complexes; most of the complexes did not evoke resistance to a lethal dose of LPS (Table II). Prior treatment with myristic acid–BSA afforded significant protection against a challenge of 15 mg of LPS/kg.

Mice treated with LPS developed resistance to a combination of lipid A-Con A complex and mithramycin that killed untreated mice. In addition, prior treatment of mice with LPS rendered them resistant to a combination of mithramycin and a complex of myristic acid–BSA or dimethyl myristamide–Con A (Table III).

Discussion. A potential source of serious error in this study is the presence of contaminating endotoxin in one or both components of the complex. Because most complexes did not display endotoxic activity, there was not enough endotoxin in either BSA alone or Con A alone to account for these results. Because β-hydroxymyristic acid–Con A complex possessed endotoxic activity, even at a dose of 25 mg/kg, and β-hydroxymyristic acid–BSA complexes lacked detectable endotoxic activity at 50 mg/kg, there was not enough endotoxin in β-hydroxymyristic acid alone to account for these results. Because both the BSA and Con A complexes of myristic acid possessed endotoxic activity, a comparable argument for myristic acid is not possible. It should be noted that BSA, Con A, myristic acid, dimethyl myristamide, or β-hydroxymyristic acid individually did not display endotoxic activity.

Results have been somewhat variable experiment-to-experiment, particularly with complexes of dimethyl myristamide. Rosen-

TABLE II. PROTECTION OF MICE FROM ENDOTOXIN BY PRIOR TREATMENT WITH PARTICULAR LIPID–PROTEIN COMPLEXES.[a]

Complex		Number of mice	Percentage killed
Lipid	Protein[b]		
None	None	40	47
Acetic acid *N*-hydroxysuccinimide ester	BSA	20	40
Acetic acid *N*-hydroxysuccinimide ester	Con A	20	30
Caprylic acid *N*-hydroxysuccinimide ester	Con A	20	35
β-Hydroxymyristic acid	BSA	40	35
β-Hydroxymyristic acid	Con A	50	40
Hydroxystearic acid	Con A	20	60
Lauric acid *N*-hydroxysuccinimide ester	Con A	20	55
Lipid A	BSA	20	10**
Lipid A	Con A	20	0**
Myristic acid	BSA	35	26*
Myristic acid	Con A	35	34
Palmitic acid *N*-hydroxysuccinimide ester	BSA	20	45
Stearic acid *N*-hydroxysuccinimide ester	BSA	20	50
Stearic acid *N*-hydroxysuccinimide ester	Con A	20	65

[a] Male ICR mice were injected ip with 50 mg of lipid–protein complex/kg on Day 1 and 10 mg/kg on Days 5, 6, and 7. On Day 8, the mice were challenged with 15 mg of *Escherichia coli* 026:B6 lipopolysaccharide (Boivin). Lethality was scored on Day 12.

[b] Con A, concanavalin A; BSA, bovine serum albumin.

* Significantly different from control group that was not treated with a lipid–protein complex at $P < 0.05$.

** Significant at $P < 0.01$.

TABLE III. PROTECTION OF MITHRAMYCIN-TREATED MICE FROM THE LETHAL EFFECTS OF LIPID–PROTEIN COMPLEXES BY PRIOR TREATMENT WITH LIPOPOLYSACCHARIDE.[a]

Lipid–protein complex[b]	Pretreated	Number of mice	Percentage dead
Lipid A–Con A (5 mg/kg)	–	20	100
Lipid A–Con A (5 mg/kg)	+	23	55*
Myristic acid–BSA (50 mg/kg)	–	25	92
Myristic acid–BSA (50 mg/kg)	+	21	48*
Dimethyl myristamide–Con A (50 mg/kg)	–	25	84
Dimethyl myristamide–Con A (50 mg/kg)	+	21	33*

[a] Male BALB/c mice were administered 1 mg of *E. coli* 026:B6 LPS/kg on Day 1, 2 mg of LPS/kg on Days 2 and 3, and 4 mg of LPS/kg on Day 4. Untreated and treated mice were administered ip 0.5 mg of mithramycin/kg and 50 mg of lipid–protein complex/kg on Day 5.

[b] BSA, bovine serum albumin; Con A, concanavalin A.

* Significantly different from the corresponding untreated control group at $P < 0.01$.

streich *et al.* (7) observed that the physical state of *N*-palmitoyl D-glucosamine was a factor in its mitogenicity. Perhaps the size of the lipid–protein complex is a determinative factor in the present experiments.

Unequivocal demonstration of endotoxic activity associated with a simple fatty acid will be of fundamental importance in furthering our understanding of the mechanism of action of bacterial endotoxin. These results indicate that the fatty acid is an important functional component of the LPS toxophore and that the remainder of the molecule may be important for solubility and for orienting the glycolipid or for exposing the toxophore in such a way that it can interact with its vulnerable receptor site. The ability to prepare substances of known composition that possess endotoxic activity should make it possible to correlate chemical structure with biological activity (7, 8).

Summary. Complexes of myristic acid and bovine serum albumin, myristic acid and concanavalin A, β-hydroxymyristic acid and concanavalin A, or dimethyl myristamide and concanavalin A are lethal for male BALB/c mice treated with mithramycin. Prior treatment of mice with myristic acid–protein complexes renders the animals resistant to a dose of bacterial endotoxin that is lethal for untreated animals. Prior treatment of mice with bacterial endotoxin renders them resistant to a combination of mithramycin and a complex of myristic acid and

bovine serum albumin or dimethyl myristamide and concanvalin A that is lethal for untreated animals. These data indicate that a fatty acid is an important functional component of the endotoxin toxophore.

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