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Isolation of a Nontoxic Lipid A Fraction Containing Tumor Regression Activity[1]

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ABSTRACT

Galanos-type endotoxin obtained from the heptose-less mutant of *Salmonella typhimurium* was converted to Lipid A by two cycles of treatment with sodium acetate, pH 4.5, at 100° and separated on a DEAE-cellulose column into several fractions (Fractions III to VII). Tumor regression studies with strain 2 guinea pigs and syngeneic line 10 hepatocellular carcinoma showed that all fractions were effective when combined with trehalose dimycolates and an additional tumor regression factor (previously designated ACP) and incorporated into oil droplets (78 to 100% cures). A low polar fraction (Fraction IV) was relatively nontoxic [the medium lethal dose for 11-day-old chick embryos inoculated i.v. ($CELD_{50}$) was more than 10 μg] and nonpyrogenic [the dose estimated to give a fever index (area under fever curve) of 40 sq cm in rabbits when 1 hr and 1° are plotted as 1 (FI_{40}) was 5 μg] as compared to the unfractionated Lipid A ($CELD_{50}$ of 0.0546 μg; FI_{40} of 0.046 μg). All other fractions were toxic and pyrogenic and caused severe endotoxic shocks when combined with *N*-acetylmuramyl-L-seryl-D-isoglutamine and injected i.v. into guinea pigs. Fraction IV plus *N*-acetylmuramyl-L-seryl-D-isoglutamine did not cause endotoxic shock. The phosphate content of Fraction IV was about one-half of that detected in the toxic fractions.

INTRODUCTION

Ribi *et al.* (12) showed that, when crude endotoxin from the heptose-less mutant of *Salmonella typhimurium* or *Salmonella minnesota* was combined with TDM[3] in oil droplets and injected directly into established tumors (line 10 hepatocellular carcinoma) in syngeneic strain 2 guinea pigs, over 90% of the animals were cured. Further study revealed that the toxicity of the endotoxin might be separable from the tumor regression activity (14). This was an important observation since endotoxic substances are not suitable to use for immunotherapy of human cancers.

We now report on the isolation of nontoxic component(s) from KDO-depleted endotoxin by DEAE-cellulose column chromatography which was highly effective in regressing line 10 tumors.

MATERIALS AND METHODS

Materials. Synthetic MDP was prepared and donated by Dr. Steve Schwartzman (18). TDM was isolated from *Mycobacterium tuberculosis* strain Aoyama B and purified by pressure elution chromatography through microparticulate silica gel (13). ACP was prepared from a crude endotoxin preparation of Chen *et al.* (3) by the method of Ribi *et al.* (14). Glucosamine and KDO were purchased from Sigma Chemical Co., St. Louis, Mo. Methyl esters of lauric (C_{12}), myristic (C_{14}), palmitic (C_{16}), stearic (C_{18}), α-hydroxymyristic, and β-hydroxymyristic acids were obtained from Applied Science Division, Laboratory Data Control, Park Ridge, Ill.

Analytical Procedures. Total phosphorus was determined by the method of Bartlett (2). KDO content was determined by the method of Osborn (9). Samples were hydrolyzed for the glucosamine and ethanolamine assays in 3 N HCl for 4 hr at 95° and for the amino acid assays in 6 N HCl for 12 hr at 95° and analyzed on a Durrum Model D-500 amino acid analyzer (Dionex Corp., Sunnyvale, Calif). The method B of Gmeiner and Martin (5) was used to prepare the fatty acids for GLC. Analytical GLC was carried out on a Packard Model 419 gas chromatograph with a glass 1.83-m x 4-mm column containing 10% Silar 10C Gas-Chrom Q (100/120 mesh) (Applied Science Division, Laboratory Data Control). A flame ionization detector was used, and the injection temperature was 170°. The column was programmed at 2.5°/min from 170-225°, and the flow rate was 60 ml of helium per min. TLC was performed on Silica Gel H (E. Merck, Darmstadt, Germany) with the solvent system of chloroform:methanol:water:concentrated ammonium hydroxide (50:41:10:1, v/v). The samples (250 μg) were applied to the plate as 1-cm streaks. Bands were visualized by spraying with potassium dichromate (0.6%) in sulfuric acid (55% by weight) reagent and charring.

Growth of Organisms and Preparation of Endotoxin. *S. typhimurium* strain G30/C21, a heptose-less *Re* mutant obtained from the late Werner Braun, was grown at 37° in a fermenter with vigorous aeration in a modified ammonium medium of Anderson (1). The medium was prepared by mixing the following salts in 1 liter of water: NaCl, 2.0 g; NH_4Cl, 1.0 g; Na_2HPO_4, 6.0 g; KH_2PO_4, 3.0 g; and $MgSO_4$, 0.1 g. Sterilized 25% (w/v) dextrose (16 ml) was then added to 984 ml of the above sterile medium and inoculated with *S. typhimurium*. The cells were harvested after 12 hr of incubation. For the preparation of cell walls, freshly harvested cells were washed once with water by resuspension and centrifugation and were suspended in aqueous 0.5% Tween 80 (polyoxyethylene sorbitan monooleate; Atlas Chemical Industries Corp., Wilmington, Del.) to a concentration of 350 mg wet weight per ml. The suspension was then processed in a Sorvall-Ribi refrigerated cell fractionator at 37,000 psi. The effluent which contained no more than

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[3] The abbreviations used are: TDM, trehalose dimycolates; KDO, 2-keto-3-deoxyoctonate; MDP, *N*-acetylmuramyl-L-seryl-D-isoglutamine; ACP, a nontoxic acetone:chloroform precipitate side fraction of endotoxin that contains an ingredient(s) needed for tumor regression in guinea pigs; GLC, gas-liquid chromatography; TLC, thin-layer chromatography; $CELD_{50}$, the medium lethal dose for 11-day-old chick embryos inoculated i.v.; FI_{40}, the dose estimated to give a fever index (area under fever curve) of 40 sq cm in rabbits when 1 hr and 1° are plotted as 1; i.d., intradermally.

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5% unbroken cells as determined by microscopic examination was centrifuged at 31,000 × *g* for 60 min to yield a supernatant fluid containing most of the soluble protoplast and a sediment consisting predominantly of cell walls. The crude cell walls were washed once with 0.5% Tween 80 and twice with water (washings involved centrifugation at 31,000 × *g* for 90 min). The crude cell walls were suspended in phosphate-buffered saline (0.15 M NaCl and 0.01 M NaH_2PO_4-Na_2HPO_4, pH 7.6; 20 mg, wet weight, per 100 ml) and treated with trypsin (0.1 μg/ml) for 3 hr at 22° with stirring and washed 3 times with water to yield the purified cell walls. The Galanos-type endotoxin was prepared from the cell walls by a published method (4). Endotoxin isolated from cell walls, in contrast to endotoxin isolated from whole cells, appeared to be "soluble" in aqueous medium as well as in organic solvents, which facilitated both its use for biological work and its fractionation by chromatographic techniques.

Preparation of Lipid A. The endotoxin (1280 mg) was suspended in 216 ml of 19.2 mM sodium acetate, pH 4.5, and incubated at 100° for 30 min (16). The reaction mixture was dialyzed against deionized distilled water and centrifuged at 48,200 × *g* for 15 min, and the residue was lyophilized. A second cycle of this treatment was performed to yield 653.7 mg of Lipid A.

DEAE-Cellulose Column Fractionation. A modified method of Rouser and Fleischer (17) for the fractionation of polar lipids on a DEAE-cellulose column was used to fractionate Lipid A. Two sets of Lipid A (315 mg each) were dissolved in 15 ml each of chloroform:methanol (4:1, v/v), applied to a 3.2- x 26-cm DEAE-cellulose column in the acetate form, and eluted with 2000 ml of the same solvent. The column was then eluted with 1000 ml each of chloroform:methanol (7:3, v/v) and methanol, followed by 2000 ml of a linear gradient of 0 to 0.5 M ammonium acetate in methanol:water (99:1, v/v). The column was finally stripped of sample by eluting with 800 ml of 1.5 M ammonium acetate in methanol:water. A very small amount of Lipid A was found in this last fraction. Twelve-ml fractions were collected and analyzed for total phosphorus. The chloroform:methanol (7:3, v/v) and methanol effluents contained very little material and were discarded. The column fractionation yielded Fractions III to VII. Each of these fractions from 2 sets was desalted by dissolving the sample in 250 ml of chloroform:methanol (2:1, v/v) and partitioning it against 100 ml of water. The lower organic layer was filtered, dried, and weighed.

Biological Assays. The endotoxicity of Lipid A fractions was measured as described by Milner and Finkelstein (8). The data are reported as (*a*) $CELD_{50}$ and (*b*) FI_{40}. To determine whether MDP enhances the susceptibility to Lipid A and causes endotoxic shock, groups of 5 strain 2 guinea pigs (350 to 550 g) were inoculated i.v. with 0.4 ml of phosphate-buffered saline (pH 7.3) containing MDP and Lipid A as combinations thereof. Animals were examined for symptoms of shock, and deaths were recorded 18 hr after inoculation (11).

Tumors and Preparation of Emulsions for Injection. The tumor model was developed at the National Cancer Institute (10). The carcinogen-induced line 10 hepatocellular carcinoma was maintained in the ascites form and transplanted (10^6 tumor cells) i.d. into 400- to 500-g syngeneic strain 2 guinea pigs. Untreated animals died in 60 to 90 days. Tumors were treated 6 days after transplantation when they were 9 to 10 mm in diameter. At this time, metastatic tumor cells are present in the regional lymph nodes (10). Test materials were associated with minute oil droplets in 0.9% NaCl solution emulsion containing 0.2% Tween 80 and about 1% light mineral oil (10 to 20 μl/mg material). They were injected in single 0.4-ml volumes directly into the tumors (15). Treated animals were observed for at least 3 months. As used here, the terms "regression" or "cure" mean complete disappearance of primary dermal tumor, no clinical evidence of metastatic disease, and rejection of contralateral challenge with 10^6 tumor cells 2 months after the original tumor was treated (7).

RESULTS

Preparation and Fractionation of Lipid A. Lipid A was prepared from a large sample of Galanos-type endotoxin by the pH 4.5 treatment which reduced the KDO content from 0.55 to 0.02 μmol/mg. This represented about 4% of the initial value. This sample was then fractionated on a DEAE-cellulose column to yield Fractions III to VII (Chart 1). These fractions were analyzed for both biological and chemical properties.

TLC of Column Fractions. As shown in Fig. 1, we could not fractionate the Galanos-type endotoxin by TLC. However, the Lipid A preparation fractionated reasonably well but showed a complex series of bands. Dark bands appeared at R_F 0.3 to 0.5, whereas light bands appeared at R_F 0.6 to 0.8. Fractions III, V, VI, and VII also showed complex series of bands appearing at R_F 0.35 to 0.55. Fraction IV was quite different in that it contained faster-moving bands which appeared at R_F 0.55 to 0.90. This fraction was clearly more lipophilic than the other fractions. TLC analysis showed that all DEAE-cellulose column fractions were still very complex mixtures.

Biological Properties of Lipid A Fractions. The starting material was highly toxic for chick embryo ($CELD_{50}$, 0.008 μg) and moderately pyrogenic for rabbit (FI_{40}, 0.155 μg). When the Lipid A was prepared and fractionated, each fraction was categorized as a high or low toxic-pyrogenic fraction (Table 1).

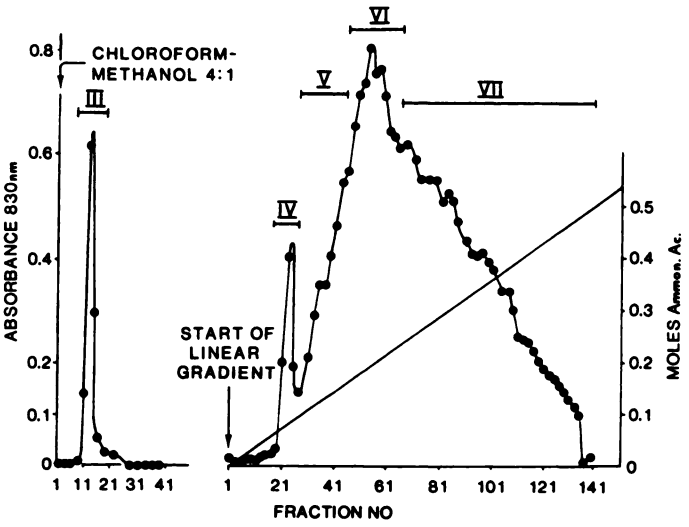

Chart 1. DEAE-cellulose column chromatography of Lipid A. Sample (315 mg) was dissolved in chloroform:methanol (4:1, v/v) and applied to a 3.2- x 26-cm DEAE-cellulose column in the acetate form. The order of elution was: (*a*) 2000 ml of chloroform:methanol (4:1, v/v); (*b*) 1000 ml each of chloroform:methanol (7:3, v/v) and methanol; and (*c*) 2000 ml of a linear gradient of 0 to 0.5 M ammonium acetate in methanol:water (99:1, v/v). Fractions of 12 ml were collected and analyzed for total phosphorus.

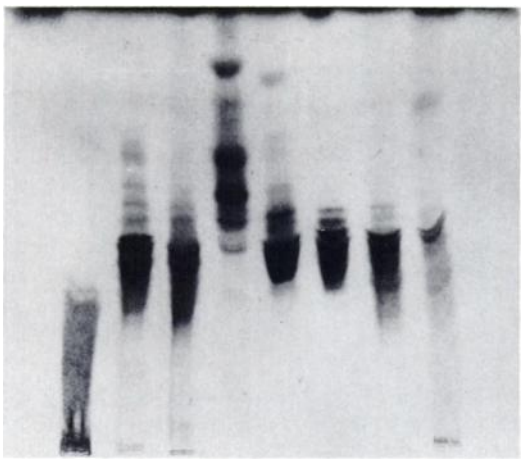

Fig. 1. TLC of Lipid A fractions from DEAE-cellulose column fractionation. Identification from *left* to *right*: *Lane 1*, endotoxin; *Lane 2*, Lipid A; *Lane 3*, Fraction III; *Lane 4*, Fraction IV; *Lane 5*, Fraction V; *Lane 6*, Fraction VI; *Lane 7*, Fraction VII; *Lane 8*, a 1.5 M ammonium acetate in methanol:water (99:1, v/v) column effluent.

Table 1

Endotoxic activity of fractions obtained by DEAE-cellulose column chromatography of Lipid A

Each of the tested fractions was solubilized in pyrogen-free 0.15 M NaCl containing 0.5% triethylamine and diluted appropriately before i.v. injection into 11-day-old chick embryos or mature rabbits.

Material tested	Lethality for chick embryos ($CELD_{50}$, μg)	Pyrogenicity for rabbits (FI_{40}, μg)
Endotoxin, untreated	0.008	0.155
Lipid A fraction		
II	0.055	0.046
III	0.465	
IV	>10	5.0
V	0.0417	0.047
VI	0.0398	0.0014
VII	0.0168	0.048

Fraction VII was most toxic ($CELD_{50}$, 0.0168 μg) and Fraction VI was most pyrogenic (FI_{40}, 0.0014 μg). Fraction IV was essentially nontoxic ($CELD_{50}$ > 10 μg) and of low pyrogenicity (FI_{40}, 5.0 μg). All fractions were active in regressing tumor when combined with TDM and ACP (78 to 100% cures; Table 2). When compared to controls, these cure rates were statistically significant.

Ribi *et al.* (11) combined the endotoxin with MDP and TDM (150 μg each) and incorporated the mixture into oil droplets. When they injected this mixture into established dermal line 10 tumors of guinea pigs, they observed that a significant number of animals died of endotoxic shock. All surviving animals suffered from severe lethargy. Therefore, they cautioned the use of MDP and endotoxin for immunotherapy of cancer. We examined the possibility that MDP in combination with the column fractions might cause endotoxic shock, and the results are presented in Table 3. At the dose levels tested, all toxic fractions in combination with MDP caused endotoxic shock in guinea pigs. In these cases, all surviving animals showed severe lethargy. In contrast, when MDP was combined with the nontoxic Fraction IV, none of the animals died and they showed no symptoms of shock.

Chemical Properties of Lipid A Fractions. The toxic Fractions III, V, VI, and VII had total phosphorus values ranging from 1.03 to 1.15 μmol/mg (Table 4). The nontoxic Fraction IV contained only 0.54 μmol phosphorus per mg. The glucosamine content was measured with the amino acid analyzer and gave somewhat lower values than expected. All values were similar (0.68 to 0.79 μmol/mg) except that of Fraction IV, which was slightly lower (0.50 μmol/mg). The ethanolamine content (not shown) of the fractions ranged from 0.05 to 0.09 μmol/mg.

The Galanos-type endotoxin contained 11.85 μg of amino acids per mg of sample (1.20%). When this preparation was treated with sodium acetate at pH 4.5 to yield Lipid A (Fraction II), the amino acid content was reduced to 0.77%. The results of the amino acid analysis of Lipid A and the DEAE-cellulose column fractions are given in Table 5. DEAE-cellulose column Fractions III and IV contained the least amounts of amino acids (0.32 and 0.45%, respectively). The results clearly showed that only trace amounts of amino acids (and thus, proteins) are present in Lipid A and all column fractions.

Normal fatty acid content which included lauric, myristic, palmitic, an unidentified acid (C_{17}), and stearic acids, ranged from 0.60 to 1.16 μmol/mg. Fraction VI had the highest normal fatty acid content. Fractions V and VII were characterized by low and high lauric acid contents, respectively (not shown). All fractions had similar hydroxy fatty acid contents. These acids consisted of both α- and β-hydroxymyristic acids of which the β-hydroxymyristic acid was the major component (97 to 99 M %).

Table 2

Regression of line 10 tumors in strain 2 guinea pigs treated with fractions obtained by DEAE-cellulose column chromatography of Lipid A, combined with TDM and ACP

Each guinea pig received a single intratumor injection of 150 μg of the untreated endotoxin or Lipid A column fraction combined with 50 μg of TDM and 150 μg of ACP in 0.4 ml of oil:Tween:saline. All fractions (III through VII) when combined with TDM without ACP were inactive in regressing tumors (0/9 in each case). All control animals given ACP plus TDM died within 60 to 90 days.

Material tested	No. of animals cured/total no. of animals treated	% of animals cured	*p* value[a]
Endotoxin, untreated	5/8	63	<0.01
Lipid A fraction			
III	7/7	100	<0.001
IV	7/9	78	<0.005
V	8/9	89	<0.001
VI	9/9	100	<0.001
VII	9/9	100	<0.001
ACP	0/9	0	NS[b]
Oil:Tween:saline	0/9	0	

[a] As determined by χ^2 contingency table analysis for difference with guinea pigs given oil:Tween:saline.
[b] NS, not significant.

Table 3

Effect of MDP and Lipid A fractions on susceptibility of normal strain 2 guinea pigs to endotoxic shock

Material inoculated[a]	No. of dead animals/total no. of animals
MDP	0/10
Endotoxin	1/9
MDP + endotoxin	8/9
MDP + Fraction III	5/5
MDP + Fraction IV	0/5[b]
MDP + Fraction VI	3/5
MDP + Fraction VII	4/5

[a] The material (150 μg each) was dissolved or suspended in phosphate-buffered saline (0.15 M NaCl and 0.01 M NaH_2PO_4-Na_2HPO_4, pH. 7.3) and inoculated i.v.
[b] These animals did not become lethargic. In all other tests where MDP was combined with the Lipid A fractions, the surviving animals became lethargic.

Table 4

Chemical analysis of the fractions obtained by DEAE-cellulose column chromatography of Lipid A

Fraction	Recovery[a] (mg)	μmol/mg sample			
		Phospho-rus	Glucosa-mine	Normal fatty acid	Hydroxy fatty acid
III	76.6	1.03	0.72	0.67	1.86
IV	30.4	0.54	0.50	0.81	1.44
V	51.9	1.03	0.73	0.60	1.98
VI	88.9	1.08	0.68	1.16	1.50
VII	142.5	1.15	0.79	0.87	1.66

[a] Total recovery including the 1.5 M ammonium acetate in methanol:water effluent was 408.8 mg (68%).

Table 5

Amino acid content of the DEAE-cellulose column fractions

Fraction II is Lipid A and Fractions III to VII are DEAE-cellulose column fractions.

Amino acid	μg amino acid/mg sample					
	II	III	IV	V	VI	VII
Aspartic acid	3.39	1.06	2.46	3.66	2.73	2.33
Serine	0.53	0.79	0.63	0.47	0.68	1.00
Glutamic acid	0.44		0.37		0.44	
Proline		0.75				
Glycine						1.09
Alanine					1.31	
Valine				1.29		0.29
Methionine					0.67	
Isoleucine	0.20	0.26		0.33	0.39	0.13
Leucine	0.33		0.79		0.59	0.66
Tyrosine	0.45				0.72	0.72
Phenylalanine	0.58			0.50		
Lysine	0.95	0.29	0.29	0.44	0.44	0.66
Histidine	0.62	0.85		0.47	0.85	0.47
Arginine	0.61					
Total	7.68	3.15	4.54	7.16	8.82	7.35
% of amino acid	0.77	0.32	0.45	0.72	0.88	0.74

DISCUSSION

Lipid A was prepared from Galanos-type endotoxin by a relatively mild procedure in which 96% of the KDO was removed. This preparation was toxic according to the chick embryo lethality test and pyrogenic when injected into rabbits. When this preparation was fractionated on a DEAE-cellulose column, the first fraction to elute from the column (Fraction III) was the highly aggregated Lipid A which was simply not adsorbed to the column. When a linear gradient of ammonium acetate in methanol:water was used, a sharp peak (Fraction IV) appeared which was followed by a series of poorly resolved fractions (V to VII).

Fraction IV differed from the other fractions in that it was relatively nontoxic and nonpyrogenic, yet in combination with TDM and ACP it caused regression of line 10 tumors comparable to the toxic and pyrogenic fractions. Moreover, it did not cause endotoxic shock in guinea pigs when combined with MDP (11).

It is presently impossible to determine why Fraction IV was nontoxic while all other fractions were toxic. One reason might be that this fraction could be devoid of the acid-labile sugar 1-phosphate group (6). Perhaps this phosphate group is responsible for the toxic nature of endotoxins. This is indicated by the low total phosphorus content in Fraction IV. We have found in preliminary experiments that treatment of the toxic column fractions with 0.1 N HCl at 100° for 15 min significantly reduced their toxicity. This separation of toxicity was probably not due to the removal of proteins since the Lipid A which was fractionated on the column contained only 0.77% amino acids. Amino acid analysis suggested that there was very little protein in any of the DEAE-cellulose column fractions. This was also supported by the fact that a ninhydrin-positive band (protein or peptide) was absent at or near the origin of TLC of Lipid A or any of the column fractions (data not shown).

Because Fraction IV is still a complex mixture, further fractionation is required. We are presently developing the methodology to fractionate Lipid A by TLC and high-performance liquid chromatography so that a meaningful structural determination can be made. Eventually, we wish to identify the structural features responsible for both antitumor activity and endotoxicity.

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