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PATHOPHYSIOLOGICAL PROPERTIES OF ENDOTOXIN
FROM 'SERRATIA MARCESCENS' 08

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RELATIONSHIP OF CHEMICAL STRUCTURE TO PATHOPHYSIOLOGICAL PROPERTIES
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A. Prancan, and L. B. Hinshaw

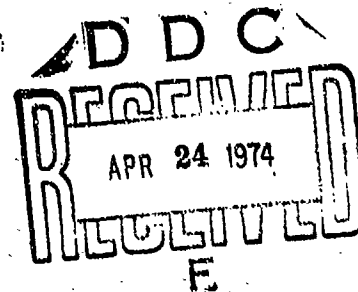
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ABSTRACT

Prior investigations in a variety of animal species have clearly demonstrated that endotoxin administration is capable of eliciting a notable series of pathophysiological events culminating in irreversible shock and death. Results from our prior studies have implicated the lipopolysaccharide portion of the endotoxin complex as the responsible agent for these events. The present work was carried out to define more precisely the chemical nature of the active site of the endotoxin. Endotoxin from S. marcescens has been degraded by aqueous phenol or acetic acid hydrolysis into a number of chemically defined structural fragments. Intravenous injections of these fragments into awake or anesthetized animals was carried out to compare their biochemical, hemodynamic and lethality relationships. Results strongly suggest that the typical pathophysiological manifestations of endotoxin shock depend on the presence of ester-linked fatty acids in the lipid moiety of the endotoxin complex.

Among a variety of biological reactions, circulatory changes culminating in irreversible shock represent the most characteristic injurious effect of intravenously injected endotoxins in mammals (1,2). Results of our preliminary studies on the relationship between chemical structure and hemodynamic properties of endotoxins indicated that the chemical site responsible for eliciting these responses resides most probably in the lipopolysaccharide portion of the endotoxin (3). To define more precisely the chemical nature of the active site, we have degraded the native endotoxin from S. marcescens 08 by hot 45% aqueous phenol or mild acetic acid hydrolysis into a number of chemically defined structural fragments (4,5) and examined their hemodynamic properties in anesthetized and unanesthetized dogs. Results of this study show that the typical hemodynamic manifestations of endotoxin shock are related to or triggered by the presence in lipid moiety of certain ester-linked fatty acids.

MATERIALS AND METHODS

Bacterial cells of the chromogenic strain Serratia marcescens 08, cultivated and harvested as described previously (6), were supplied by General Biochemicals, Chagrin Falls, Ohio.

Isolation of Whole Endotoxin and Preparations of Its Fragments

Wet cells washed with distilled water were extracted with 5% trichloroacetic acid according to a modified procedure of Boivin et al. (7). The detailed procedure including the extraction of nucleic acid-free endotoxin preparation with chloroform-methanol (2:1, v/v) was described previously (8).

The lipopolysaccharide fragment was isolated from the whole endotoxin by treatment with hot 45% aqueous phenol according to the method of Westphal et al. (9). Lipid A was prepared by mild acid hydrolysis of lipopolysaccharide or conjugated protein (0.1 N HCl, 100°, 30 minutes) as described previously (5,6). The isolation of the O-specific side chain, conjugated protein and pronase core of conjugated protein was carried out according to the procedures described by Wober and Alaupovic (5).

Determinations of neutral sugars, D-glucosamine, fatty acids and amino acids of whole endotoxin and its fragments were carried out as previously described (5,6,8).

Deesterification of Whole Endotoxin

The removal of ester-bound fatty acids from whole endotoxin was performed by hydroxylaminolysis (10). One-half gram of whole endotoxin was suspended in the hydroxylamine solution and the mixture was stirred under nitrogen for 20 minutes. The deesterified, insoluble endotoxin was then removed by low speed centrifugation and washed successively with 0.1 N acetic acid in 95% ethanol, acetone and 95% ethanol. The washed, deesterified endotoxin was dissolved in 100 ml distilled water and the solution was centrifuged at 40,000 rpm (105,000xg) for 3 hours. The clear supernate contained the deesterified endotoxin; a small amount of sediment was identified as intact endotoxin. The supernate was dialyzed against distilled water and lyophilized. To ensure a complete deesterification, the hydroxylaminolysis of lyophilized product was repeated according to the above procedure.

The deesterified endotoxin was dissolved in 0.15 M phosphate buffer, pH 7.0, and applied to a Sepharose 4B column (2.5 x 100 cm) equilibrated with the same buffer. The elution pattern was monitored by absorbance at 280 nm and by determining the carbohydrate content of 5 ml fractions by the phenol-sulfuric acid method. The elution volume of the major carbohydrate positive fraction was 220-280 ml. A small, carbohydrate-negative fraction eluted at 290-320 ml was discarded (the weight of lyophilized material was less than 5% of the applied deesterified endotoxin). The major fraction was dialyzed against distilled water, lyophilized, redissolved in 0.005 M borate buffer and applied to a DEAE-cellulose column (2 x 30 cm). The column was developed with a linear gradient of NaCl between 0 and 1 M. A single peak of purified deesterified endotoxin was eluted by approximately 0.15 M NaCl.

Lethality for Mice (LD₅₀)

Toxicity studies were carried out with male Balb-C inbred mice (Texas Inbred Mouse Co., Houston, Texas) according to the method described previously (5). The whole endotoxin and its fragments were solubilized in 0.05 M Tris buffer containing 0.5% sodium dodecyl sulfate (Tris-SDS buffer), pH 7.6.

Hemodynamic Effects in Dogs

Experiments were carried out with anesthetized and unanesthetized adult mongrel dogs. In the first phase of this study 26 dogs were anesthetized with sodium pentobarbital, 30 mg/kg, and the hemodynamic parameters were measured during a 4 hour period. Catheters were placed in the femoral artery and vein for measurement of mean systemic

arterial pressure and for collection of blood samples, respectively. Laparotomy was performed and a catheter was introduced into the portal vein through an accessory splenic vein for measuring the portal venous pressure. Mean systemic arterial pressure, portal vein pressure, heart rate, pH, and hematocrit were recorded at specified times after intravenous injections of whole endotoxin and its fragments according to previously described techniques (11). Endotoxin and its fragments were injected in doses causing shock (2 mg/kg) in order to make comparisons of the degree of severity of shock on a weight basis, and animals surviving 4 hours after injections were sacrificed. Volumes injected were less than 2 cc/kg.

To avoid any possible interfering effect of anesthetics and to obtain a longer time interval for observations, the second phase of this study was conducted with unanesthetized animals. Initially, 48 dogs were anesthetized with sodium pentobarbital, 25 mg/kg. A lateral incision was made over the jugular vein under sterile conditions. The carotid artery was freed from surrounding tissue and cannulated with silastic tubing, varying in size to fit the different vessels. The catheters were constructed with a small polyethylene collar that could be inserted into the artery along with the tubing. Ties were then placed on both sides of the collar to anchor the catheter in place. After the incision was closed the tubing was filled with sodium heparin, 1000 units/ml, stoppered and taped to the side of the dog's neck. Daily flushings with heparinized saline were used to keep the catheters free of blood clots. After a recovery period of at least one day the dogs were restrained in a special device with sling supports so they could rest in a comfortable upright position

for an initial 2 hour study (12). The dogs were connected to a P23Db Statham pressure transducer via the indwelling carotid catheter for measuring the heart rate and mean systemic arterial pressure. Whole endotoxin and its various fragments were injected intravenously in doses causing shock and reflecting the LD₅₀ values of these preparations in mice. The injected dose of whole endotoxin was 0.3 mg/kg, conjugated protein 6 mg/kg, conjugated protein, pronase core 3 mg/kg, lipid A 5 mg/kg, and O-specific side chain and deesterified whole endotoxin 25 mg/kg. Volumes of doses injected were 1 cc/kg for buffer, saline, side chain, and lipid A, and less than 6 cc/kg for whole endotoxin, conjugated protein, pronase core and deesterified whole endotoxin. Blood samples taken at zero time, 120 min, and 2 days after injection were used for measuring the hematocrit and pH according to the previously described procedures (11,12). Serum glutamic pyruvic transaminase (SGPT) and serum glutamic oxalacetic transaminase (SGOT) were also carried out as previously described (12). A continuous pressure recording was made on a two-channel Sanborn recorder. Heart rates were recorded every 15 minutes. Dogs surviving for 2 days were sacrificed and recorded as survivors.

RESULTS

Chemical Composition of Whole Endotoxin and Its Fragments (Table 1)

Purified whole endotoxin isolated by a 5% trichloroacetic acid extraction of S. marcescens 08 is a macromolecular compound consisting of carbohydrate, lipid and protein moieties. Small amounts of contaminating free O-specific side chain and protein moiety can be

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13. ABSTRACT Prior investigation in a variety of animal species have clearly demonstrated that endotoxin administration is capable of eliciting a notable series of pathophysiological events culminating in irreversible shock and death. Results from our prior studies have implicated the lipopolysaccharide portion of the endotoxin complex as the responsible agent for these events. The present work was carried out to define more precisely the chemical nature of the active site of the endotoxin. Endotoxin from <u>S. marcescens</u> has been degraded by aqueous phenol or acetic acid hydrolysis into a number of chemically defined structural fragments. Intravenous injections of these fragments into awake or anesthetized animals was carried out to compare their biochemical, hemodynamic and lethality relationships. Results strongly suggest that the typical pathophysiological manifestations of endotoxin shock depend on the presence of ester-linked fatty acids in the lipid moiety of the endotoxin complex.			

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detected by immunological technique (4). Whole endotoxin was characterized by 23% neutral sugars, 12.2% D-glucosamine, 20.1% fatty acids, and 11% amino acids.

The hot aqueous phenol treatment of whole endotoxin cleaved a sensitive linkage within the lipid moiety and resulted in the formation of two very typical fragments recognized as simple protein and lipopolysaccharide (4). In comparison with its parent compound, the lipopolysaccharide was characterized by a higher content of carbohydrate (33%) and D-glucosamine (33%) and a lower content of fatty acids (9%). The decreased fatty acid content of lipopolysaccharide was caused not only by a selective removal of a part of the lipid moiety but also by the partial hydrolysis of the ester-linked fatty acids during phenol treatment of whole endotoxin (13). The lipopolysaccharide only contained traces of amino acids. The chemical composition of conjugated protein attested to the fact that mild acetic acid hydrolysis of whole endotoxin results in an almost quantitative and selective cleavage of bonds between the lipid moiety and polysaccharide core (5). The cleavage of the major portion of O-specific side chain and core is reflected in a low content of neutral sugars (3.1%) and D-glucosamine (9.4%) and a high content of fatty acids (46%) and amino acids (35.3%). Pronase treatment of conjugated protein resulted in a product characterized by increased relative content of the fatty acids (53.2%) and decreased content of amino acids (12.9%). Lipid A contained a high percentage of fatty acids (68.9%) and D-glucosamine (24%) and only trace amounts of neutral sugars (2%) and amino acids (1%). Hydroxylaminolysis of whole endotoxin caused a selective

cleavage of ester-bound fatty acids. The infrared spectrum showed that the remaining small amount of fatty acids (1.8%) were bound through amide linkages. O-specific side chain was free of fatty acids and amino acids. Its structure has been reported (8).

Whole endotoxin, lipopolysaccharide, conjugated protein and lipid A were highly toxic in mice (5). On the other hand, the O-specific side chain and the deesterified whole endotoxin showed no toxic effects in doses up to 100 mg/kg. Increased solubility of lipid A in Tris-SDS buffer eliminated the necessity to solubilize lipid A by complexing with protein carriers (14).

Hemodynamic Effects in Dogs

Results in Table 1 showed that injection to anesthetized dogs of whole endotoxin or any of its three fragments, i.e., lipopolysaccharide, conjugated protein and lipid A, elicited hemodynamic changes resulting in shock. Significant decreases in mean arterial pressure and elevations in portal vein pressure were accompanied by bradycardia and a progressive development of acidosis and hemoconcentration. Animals injected with Tris-SDS buffer showed no significant changes in any of these parameters.

In the second phase of this study unanesthetized dogs were injected with whole endotoxin, conjugated protein and its promase core, lipid A, O-specific side chain and deesterified whole endotoxin. The results are presented in Table 2. Two types of controls were carried out. Saline was injected to rule out any possible interfering effect of volume and the Tris-SDS buffer was tested for the possible contributions of anionic detergent to the toxicity of endotoxic preparations. It was noted that neither the saline nor Tris-SDS buffer

had any significant effect on hemodynamic parameters. Injection of either the whole endotoxin, conjugated protein, pronase core or lipid A caused a marked decrease in the mean arterial pressure and pH values, and an increase in the heart rates and hematocrits. In contrast to these results, the administration of O-specific side chain or deesterified whole endotoxin had no significant effect on any of the hemodynamic parameters.

Whole endotoxin and all its fragments, including the O-specific side chain, caused an elevation of serum glutamic oxalacetic and glutamic pyruvic transaminases (Table 3). Deesterified whole endotoxin had no effect on levels of either of these two enzyme activities.

Administration of whole endotoxin, conjugated protein and lipid A resulted in high mortality rates (Table 4). However, no deaths were recorded after injection of O-specific side chain or deesterified whole endotoxin.

DISCUSSION

Comparative chemical studies on endotoxic products isolated either by 45% aqueous phenol, 1% acetic acid or 5% trichloroacetic acid treatment of Gram-negative bacteria indicated that each of these commonly used procedures caused degradation of endotoxins into some very characteristic fragments. The 45% aqueous phenol treatment of bacterial cells cleaved a weak linkage within the lipid moiety and resulted in the degradation of endotoxin into two fragments designated as simple protein and lipopolysaccharide (4). As a result of this degradation, both simple protein and lipopolysaccharide retained a portion of the lipid moiety. Prolonged treatment with aqueous phenol also resulted in the partial cleavage of O-specific side chain from

the polysaccharide core and ester-linked fatty acids in the lipid moiety (13). Although the lipopolysaccharide fragment only contained a portion of the lipid moiety, it exhibited high levels of toxicity. The acetic acid extraction caused a complete fragmentation of endotoxin into conjugated protein, O-specific side chain and polysaccharide core (5). Conjugated protein consisted of intact protein and lipid moieties, and negligible amounts of polysaccharide core. The toxicity of conjugated protein was equal to or greater than that of lipopolysaccharide.

Among key fragments, both lipopolysaccharide and simple protein contain a portion, while conjugated protein retains the entire lipid component. The lipid A preparations isolated from lipopolysaccharide (6) or protein fragments (5) by mild acid hydrolysis are mixtures of various degradation products, mostly partially deacylated diglucosamine units, of intact lipid moiety.

Results of the present investigation showed that anesthesia had no effect on the hemodynamic properties of endotoxic compounds. The typical changes in hemodynamic parameters, transaminase levels (12) and mortality rates were only observed with whole endotoxin and fragments which contained at least a portion of the lipid moiety. It is noteworthy that the toxicity and hemodynamic properties of conjugated protein differed very little from those of lipopolysaccharide. The possible effect on hemodynamic parameters of the protein moiety was excluded, because a protein-free lipopolysaccharide was biologically as effective as whole endotoxin. Similarly, the O-specific side chain, the carrier of serological specificity of bacterial cells, had no effect on measured hemodynamic parameters. These results pointed

to the lipid moiety as the primary site responsible for biological activity. This possibility was tested with a lipid A preparation isolated from lipopolysaccharide fragment and solubilized in 0.5% sodium dodecyl sulfate. It was clearly demonstrated that lipid A, due to an increased solubility in sodium dodecyl sulfate, was a highly active inducer of hemodynamic reactions characteristic of endotoxin shock. Since lipid A does not represent an intact lipid moiety, it is obvious that structural features necessary for biological activity were preserved during its isolation from lipopolysaccharide by mild acid hydrolysis. Results of an experiment with deesterified whole endotoxin indicated the ester-bound fatty acids as functional groups directly responsible for hemodynamic properties of endotoxin.

Elevations of serum levels of transaminases observed in the present study are indicative of both early and sustained injury or depression of function in cardiac or hepatic tissues. Similar increases have been reported in several disease states (15-17) and following administration of live E. coli organisms (12).

It has recently been demonstrated that lipid A solubilized by complexing with bovine serum albumin is the active component responsible for complement inactivation (18), pyrogenicity (14), toxicity (14), and mitogenicity (19) of endotoxins from Salmonella and Escherichia coli. Results of present study show that also hemodynamic changes culminating in irreversible shock are elicited by lipid moiety as the carrier of active components or structures of endotoxin molecules. Furthermore, solubilization of lipid A and other lipid-containing endotoxin fragments in 0.5% sodium dodecyl sulfate solutions exposes their "toxic conformation" (14) and renders them biologically active

as efficiently as complexing with protein carriers (14). The obvious parallelism in structure-function relationship between hemodynamic properties, pyrogenicity, complement inactivation and mitogenicity shows clearly that all these effects of endotoxin reside in and are triggered by the same structural component, i.e., ester-bound fatty acids of the lipid moiety.

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TABLE 1

Effect of Endotoxin and Some of Its Fragments on the Hemodynamic Parameters in Anesthetized Dogs*

Time (min post- injection)	Number of Animals	Mean Systemic Arterial Pressure (mm Hg)	Portal Venous Pressure (mm Hg)	Heart Rate (beats/min)	Hematocrit (% RBC)	pH
<u>Tris-SDS Buffer</u>						
0	6	132 ± 6	6 ± 1	756 ± 26	39 ± 3	7.33 ± .03
120		139 ± 6	6 ± 1	162 ± 21	41 ± 2	7.73 ± .04
240		128 ± 3	7 ± 1	173 ± 18	43 ± 2	7.35 ± .03
<u>Whole Endotoxin</u>						
0	5	128 ± 5	10 ± 1	140 ± 9	45 ± 2	7.21 ± .04
120		48 ± 5	12 ± 2	134 ± 17	54 ± 3	6.98 ± .07
240		65 ± 16	11 ± 4	122 ± 13	57 ± 3	6.87 ± .13
<u>Lipopolysaccharide</u>						
0	5	123 ± 4	9 ± 1	140 ± 13	38 ± 2	7.29 ± .05
120		64 ± 9	13 ± 1	104 ± 12	48 ± 2	7.07 ± .04
240		92 ± 7	13 ± 2	130 ± 8	53 ± 2	7.12 ± .06
<u>Conjugated Protein</u>						
0	5	115 ± 10	9 ± 1	170 ± 22	37 ± 2	7.31 ± .02
120		58 ± 16	11 ± 1	141 ± 22	44 ± 4	7.01 ± .08
240		88 ± 25	12 ± 2	62 ± 18	49 ± 4	6.95 ± .11
<u>Lipid A</u>						
0	5	127 ± 5	10 ± 1	166 ± 20	34 ± 2	7.30 ± .02
120		41 ± 2	11 ± 2	124 ± 9	50 ± 2	6.80 ± .03
240		55 ± 10	10 ± 2	92 ± 14	52 ± 1	6.81 ± .03

*M±SE; whole endotoxin; lipopolysaccharide, conjugated protein and lipid A were solubilized in 0.05 M Tris buffer containing 0.5% sodium dodecyl sulfate.

Effect of Endotoxin and Its Fragments on the Hemodynamic Parameters in Unanesthetized Dogs*

Time (min post- injection)	Number of Animals	Mean Systemic Arterial Pressure (mm Hg)	Heart Rate (beats/min)	Hematocrit (% RBC)	pH
0	6	101.6 ± 8.33	140.8 ± 11.14	34.3 ± 2.0	7.44 ± 0.01
120	6	100.8 ± 9.78	118 ± 12.33	33.6 ± 2.8	7.44 ± 0.01
2 days	4 [†]	103.7 ± 5.54	146.3 ± 22.11	34.5 ± 5.4	7.45 ± 0.01
0	5	121 ± 3.32	102.2 ± 14.26	38.1 ± 2.4	7.43 ± 0.01
120	5	120 ± 6.89	106 ± 10.3	38.5 ± 2.3	7.43 ± 0.02
2 days	3 [†]	105 ± 8.66	136.6 ± 27.3	37 ± 1.5	7.42 ± 0.01
0	6	105.3 ± 2.60	107.6 ± 7.0	36.9 ± 1.6	7.42 ± 0.02
120	4 (2 deaths)	65.5 ± 17.85	148.5 ± 21.4	45 ± 4.0	7.36 ± 0.04
2 days	1 (5 deaths)	110	102	42.5	7.29
0	6	104.2 ± 4.73	108.3 ± 13.8	36.6 ± 1.3	7.42 ± 0.01
120	5 [†]	67 ± 20.77	141.3 ± 21.9	53.5 ± 2.2	7.24 ± 0.04
2 days	No survivors				
0	6	120.8 ± 5.39	121.7 ± 13.7	38 ± 1.9	7.41 ± 0.01
120	5 [†]	83 ± 11.79	139.6 ± 29.3	47.7 ± 5.5	7.33 ± 0.03
2 days	No survivors				

TABLE 2 (cont.)

		Lipid A					
	7	120	± 5.77	132.1 ± 14.8	35	± 1.2	7.42 ± 0.02
120	4 (3 deaths)	91	± 18.12	156.2 ± 13.9	48.8	± 2.9	7.21 ± 0.21
2 days	1 (6 deaths)	105		150	42		7.44
					<u>O-Specific Side Chain</u>		
0	5	103	± 3.39	118.6 ± 6.3	37	± 1.1	7.43 ± 0.02
120	5	100	± 7.07	124	± 25.2	38.5 ± 3.8	7.42 ± 0.02
2 days	5	98	± 5.61	128.4 ± 14.2	36.9	± 3.1	7.44 ± 0.02
					<u>Deesterified Whole Endotoxin</u>		
0	6	110	± 4.08	105	± 5.2	30.5 ± 1.9	7.43 ± 0.01
120	6	107	± 5.44	97.5	± 7.9	38.2 ± 0.9	7.43 ± 0.02
2 days	6	112.5	± 4.79	106.7	± 4.7	37.6 ± 1.6	7.41 ± 0.02

4455; whole endotoxin, lipopolysaccharide, O-specific side chain and deesterified whole endotoxin were injected as suspensions in 0.9% NaCl. Conjugated protein and its pronase core and Lipid A were solubilized in 0.05 M Tris buffer containing 0.5% sodium dodecyl sulfate.

two deaths occurred at indicated time periods. Hemodynamic measurements were not recorded due to technical difficulties.

TABLE 3

Effect of Whole Endotoxin and Its Fragments on Serum Glutamic Oxalacetic
and Serum Glutamic Pyruvic Transaminases*

Time	Number of Animals	Serum Glutamic Oxalacetic Transaminase (units)	Serum Glutamic Pyruvic Transaminase (units)
<u>Saline</u>			
0	4	29.7 ± 4.71	33.0 ± 3.50
120	4	24.7 ± 5.14	33.4 ± 3.52
2 days	3	28.6 ± 4.89	37.9 ± 10.29
<u>Tris-SDS Buffer</u>			
0	5	27.4 ± 3.89	36.9 ± 10.71
120	5	34.0 ± 6.16	36.1 ± 4.39
2 days	4	32.6 ± 4.69	42.9 ± 5.76
<u>Whole Endotoxin</u>			
0	6	22.5 ± 4.44	23.6 ± 3.75
120	4	294.5 ± 107.82	91.5 ± 32.82
2 days	1	44.5	24.75
<u>Conjugated Protein</u>			
0	6	24.7 ± 3.62	28.5 ± 5.13
120	6	281 ± 80.3	149.3 ± 65.46
2 days	0	No survivors	No survivors

TABLE 3 (cont.)

		Conjugated Protein, Pronase Core	
		26.1 ± 4.56	36.3 ± 5.60
0	6		
120	5	257.4 ± 75.9	81.2 ± 19.95
2 days	0	No survivors	No survivors
		<u>Lipid A</u>	
0	7	23.3 ± 5.04	30.2 ± 4.78
120	5	260.0 ± 82.7	135.6 ± 59.26
2 days	3	86.3 ± 36.0	86.3 ± 36.09
		<u>O-Specific Side Chain</u>	
0	4	28.9 ± 7.14	47.9 ± 13.88
120	4	241.9 ± 100.16	85.8 ± 27.71
2 days	4	314.9 ± 144.47	455.7 ± 343.38
		<u>Deesterified Whole Endotoxin</u>	
0	6	25.3 ± 3.09	23.6 ± 2.43
120	6	34.4 ± 10.68	34.4 ± 6.96
2 days	5	33.7 ± 13.53	43.6 ± 12.02

M±SE

TABLE 4

Effect of Endotoxin and Its Fragments on the Mortality in Dogs*

Preparations	Number of deaths/ Total number of animals
Saline solution	0/6
Tris-SDS buffer	0/5
Whole endotoxin (0.3 mg/kg)	5/6
Conjugated protein (6 mg/kg)	6/6
Conjugated protein, pronase core (3 mg/kg)	6/6
Lipid A (5 mg/kg)	6/7
O-specific side chain (24 mg/kg)	0/5
Deesterified whole endotoxin (25 mg/kg)	0/6

*Survival is for 2-day period.