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Biological Activities of Synthetic Lipid A Analogs: Pyrogenicity, Lethal Toxicity, Anticomplement Activity, and Induction of Gelation of *Limulus* Amoebocyte Lysate

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Chemically synthesized lipid A analogs were investigated for several endotoxic activities, including pyrogenicity, lethal toxicity, anticomplement activity, and the capacity to gelate *Limulus* amoebocyte lysate in comparison to natural lipid A. The synthetic preparations contained D-glucosamine or D-glucosamine-β-1,6-D-glucosamine disaccharide substituted by ester- and amide-bound hydroxylated or non-hydroxylated fatty acids and by phosphate groups in different combinations. Some preparations which were insoluble in water were succinylated and thus rendered more soluble. Strong biphasic pyrogenic responses with a maximal increase in body temperature of 1 to 2°C were obtained with 50 μg/kg doses of 3 disaccharide preparations of 15 tested. With two preparations (50 μg/kg) moderate pyrogenicity with monophasic fever curves and a maximal temperature increase of about 0.6°C was obtained. Lethal toxicity tests were carried out in galactosamine-sensitized mice. Of 15 synthetic preparations, 4 exhibited lethal toxicity under these conditions. The effective doses of the lipid A analogs in both in vivo tests were, however, several hundred times higher than those of bacterial lipid A. For the activities in vivo, hydroxyacyl residues seemed to be important. Anticomplement activity was demonstrable in seven preparations, one of which expressed an activity comparable to that of lipid A. Preparations containing non-hydroxylated fatty acids seemed to be most active in this test. None of the synthetic preparations was found to exhibit gelation activity for *Limulus* amoebocyte lysate when tested in doses up to 0.4 μg, whereas bacterial free lipid A was active in doses of about 2 pg. None of the monosaccharide derivatives exhibited any of these activities.

Lipopolysaccharides (LPS) are integral constituents of the outer membrane of gram-negative bacteria. They represent highly active toxins, the endotoxins, and are endowed with an overwhelming spectrum of biological activities expressed both in vivo and in vitro. LPS are amphipathic molecules consisting of a polysaccharide portion and a lipid, termed lipid A. The lipid A component represents the biologically active principle of LPS, and free lipid A, as obtained after acetic acid treatment of LPS, shows most of the endotoxic activities of LPS (12).

The recent identification of the chemical structure of lipid A (E. Th. Rietschel, U. Zähringer, H. W. Wollenweber, K. Tanamoto, C. Galanos, O. Lüderitz, S. Kusumoto, and T. Shiba, *in* A. Tu, W. H. Habig, and M. C. Hardegree, ed., *Handbook of Natural Toxins*, vol. II, in press) has prompted its chemical synthesis (7–10, 14, 20). Today, a number of synthetic preparations (lipid A analogs) are available which resemble lipid A structurally. We investigated such preparations for typical endotoxic activities to identify structure-activity relationships, to recognize minimal structure requirements for biological effects, to separate if possible specific activities, and finally, to prove the proposed structure of bacterial lipid A.

In this study the pyrogenic activity, lethal toxicity, anticomplement activity, and the capacity to gelate *Limulus* amoebocyte lysate of the synthetic lipid A analogs were tested. Lethal toxicity tests were performed in mice pretreated with D-galactosamine-hydrochloride, which induces a state of high susceptibility to the lethal action of endotoxin. This test system, therefore, represents a very sensitive method for measuring the lethal toxicity of endotoxin preparations. Some of the synthetic preparations were tested in the form of more soluble succinyl derivatives.

MATERIALS AND METHODS

Free lipid A. The LPS of a *Salmonella minnesota* Re mutant (strain 595), extracted by the phenol-chloroform-petroleum ether method (5), was treated with 1% acetic acid at 100°C for 30 min (6). Precipitated free lipid A was washed with acetic acid, followed by electrodialysis and conversion into the triethylammonium salt form (3).

Synthetic lipid A analogs. The chemical synthesis of the preparations used in this study has been described previously (7–9). Some of the synthetic (phosphate-containing) preparations could be solubilized or finely suspended in water after conversion into the triethylammonium salt by shaking in 1 N HCl at 0°C for 2 to 5 min, followed by centrifugation, washing, suspension in water, ultrasonication, and neutralization with triethylamine.

Succinylation of lipid A and synthetic preparations (16, 17). The suspension, containing 5 mg of sample (dried over P_2O_5 in a desiccator), 100 mg of succinic anhydride, and 90 μl of pyridin (freshly distilled over KOH), was heated in pyrogen-free ampoules at 60°C for 3 h. The mixture was poured into water (2 ml; 4°C) and transferred into dialysis tubes which had been washed with pyrogen-free water (1 liter; 1 h; 60°C). Dialysis was performed against water (twice 1 liter; 12 h; 4°C), followed by dialysis against 0.05% triethylamine (1 liter; 4°C). The succinylated preparations were lyophilized.

Pyrogen assay. Chinchilla-Bastard rabbits, weighing between 1.7 and 2.3 kg and bred under specific-pathogen-free

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conditions, were used. Pyrogenicity was tested in an air-conditioned room as described previously (15). Three animals were used for each dose. They were injected intravenously with 2 ml of test sample dissolved in pyrogen-free phosphate-buffered saline (PBS). Fever was measured rectally using thermistor probes connected to a recording temperature-measuring device (Hartmann und Braun, Frankfurt, Federal Republic of Germany) and expressed as the increase in temperature (ΔT) at the two fever peaks, appearing 1 and 2.5 h after injection of the test samples.

Lethality tests. Lethality tests were performed by the method described previously (2). Male 10-week-old C57BL/6 mice were obtained from the breeding stock of the Max-Planck-Institut, Freiburg, Federal Republic of Germany. In all cases the animals were injected intraperitoneally with 10 mg of D-galactosamine-hydrochloride (purchased from C. Roth DHG, Karlsruhe, Federal Republic of Germany) in 0.5 ml of pyrogen-free PBS. The samples to be tested were either mixed and injected with the galactosamine solution intraperitoneally (total volume, 0.5 ml) or injected separately by the intravenous route immediately after the administration of galactosamine. In the latter case, the samples were dissolved in 0.2 ml of pyrogen-free water.

Complement fixation test (4). Fresh guinea pig serum was obtained from the Hygiene Institute, Freiburg, absorbed with sheep erythrocytes, and stored at −80°C. Erythrocytes were sensitized as follows: 0.2 ml of packed erythrocytes was suspended in 4 ml of PBS, mixed with 10 μl of amboceptor, and incubated at 37°C for 15 min. The cells were then washed three times with PBS and suspended in 10 ml of Veronal buffer. Different amounts of test samples in 50 μl of distilled water were mixed with 50 μl of guinea pig serum. The mixtures, after incubation for 1 h at 37°C, were diluted to 150 μl with Veronal buffer. From each solution 8 μl was added to 1 ml of the same buffer plus 0.5 ml of a suspension of sensitized erythrocytes and incubated at 37°C for 1 h. After centrifugation the absorbancy of the supernatants was measured at 546 nm. The amount of complement used leads to 90% lysis of the added erythrocytes in the absence of lipid A. Anticomplement activity was expressed as the amounts (micrograms) of test samples which inhibit complement activity by 50%.

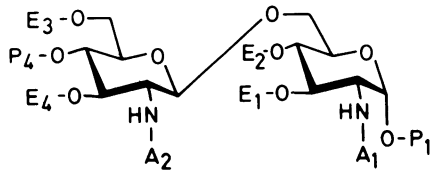
***Limulus* assay.** *Limulus* amoebocyte lysate (Pyrotell) was obtained from Associates of Cape Cod, Inc., Woods Hole, Mass., and the test was performed as previously described (22), with some modifications. Pyrogen-free flat-bottomed multiwell plates (Falcon Plastics, Oxnard, Calif.) were used, and 30 μl of test sample and 20 μl of *Limulus* amoebocyte lysate were incubated at 37°C for 1 h. The mixtures were examined for gelation by tilting the plates to 45° and tipping the side of the plate.

RESULTS

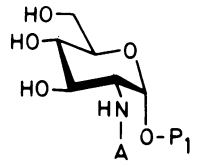
The structures and designations of the synthetic lipid A analogs, representing disaccharide and monosaccharide derivatives, are shown in Fig. 1 (for structural details of bacterial *Salmonella* lipid A, see the legend to Fig. 1). A number of the synthetic preparations containing phosphate groups could be solubilized directly in water (Table 1). Other preparations were found to be highly hydrophobic and insoluble in water. They were therefore converted into succinylated derivatives and thus rendered more water soluble. For comparison, natural lipid A was also succinylated.

Pyrogenicity of free original and succinylated lipid A. Free lipid A from *S. minnesota* Re 595 and its succinylated derivative were tested side by side with different doses for pyrogenic activity. As shown in Fig. 2, original and modified lipid A expressed high pyrogenicity, indicating that succinylation had not altered the pyrogenic activity.

Pyrogenicity of the synthetic preparations. As an example,



Sample number	Fatty acids E_{1-3} (E_4=H)	Fatty acids $A_{1,2}$	Phosphate P1	Phosphate P4
301	C14	C14		
304	C14	C14		P4
303	C14	C14	P1	
305	C14	C14	P1	P4
301	C14	C14-OH		
321	C14	C14-OH		P4
316	C14	C14-OH	P1	
317	C14	C14-OH	P1	P4
311	C14-OH	C14-OH		
312	C14-OH	C14-OH		P4
314	C14-OH	C14-O-(C14)		
315	C14-OH	C14-O-(C14)		P4



Sample number	Fatty acid A	Phosphate P1
318	C14-OH	P1
319	C14-OH	β-P1
313	C14-O-(C14)	

FIG. 1. General structure of the synthetic lipid A analogs, disaccharide, and monosaccharide derivatives. The substitutions are: E and A, ester- and amide-linked fatty acids; P_1 and P_4, phosphate groups at positions 1 (α, if not stated otherwise) and 4′. C_{14}, Tetradecanoyl; C_{14}-OH, (R)-3-hydroxytetradecanoyl; C_{14}-O-(C_{14}), (R)-3-tetradecanoyloxytetradecanoyl. *Salmonella* lipid A contains the following substituents: P_1; P_4; A_1, (R)-3-hexadecanoyloxytetradecanoyl; A_2, (R)-3-dodecanoyloxytetradecanoyl; E_1, (R)-3-hydroxytetradecanoyl; E_4, (R)-3-tetradecanoyloxytetradecanoyl; E_2 and E_3, H (Rietschel et al., in press).

TABLE 1. Pyrogenicity of synthetic lipid A analogs

Sample no.	Sample tested[a]	Solubility[b]	Dose (μg/kg)[c]	Temp increase (ΔT°C)[d]	
				Peak I (1 h)	Peak II (2.5 h)
301	succ	++	50	0	0
304	orig	−	50	0	0
	succ	++	50	0	0
303	orig	+	50	0	0
305	orig	+	50	0	0
302	orig	−	50	0	0
	succ	++	5	0.8	0
	succ	++	50	1.2	1.8
321	orig	+	50	0.6	0
316	orig	+	50	0.6	0
	succ	++	1	0.6	0
	succ	++	5	1.0	1.2
	succ	++	50	1.3	1.9
317	orig	++	1	0	0
	orig	++	5	0.6	0
	orig	++	10	0.7	0
	orig		50	1.1	1.0
311	succ	++	50	0.6	0
	succ	++	100	0.8	0
312	succ	++	50	0	0
314	orig	−	50	0	0
315	orig	−	150	0	0
318	orig	++	50	0	0
319	orig	++	50	0	0
313	succ	++	50	0	0

[a] orig, Original; succ, succinylated preparation.
[b] ++, Solution; +, fine suspension; −, particles.
[c] Intravenous.
[d] Fever peaks I and II (see Fig. 2).

Fig. 3 shows the fever response to original and succinylated preparation 316 after injection of 1, 5, and 50 μg/kg. The pattern of the fever curves is very similar to those obtained with free lipid A. However, the doses needed for similar

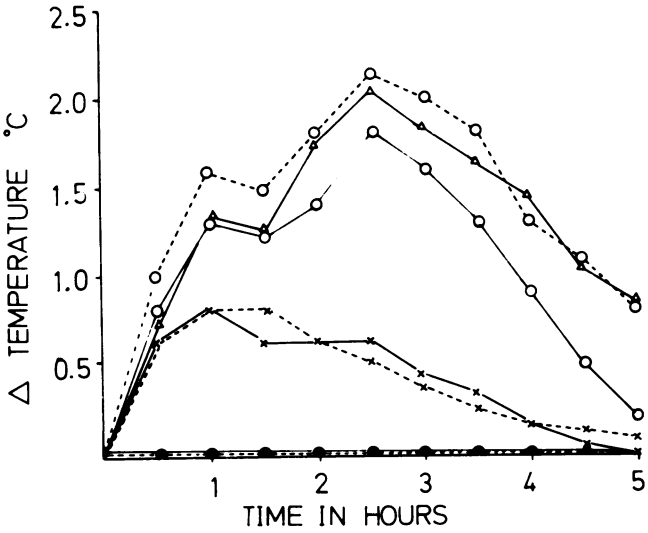


FIG. 2. Fever response to free lipid A from *S. minnesota* Re 595 and its succinylated derivative. △, 0.1 μg/kg; ○, 0.05 μg/kg; x, 0.01 μg/kg; ●, 0.001 μg/kg. Solid line, free lipid A; dashed line, succinylated lipid A. With each dose, three rabbits were tested, and the mean temperature rise was calculated.

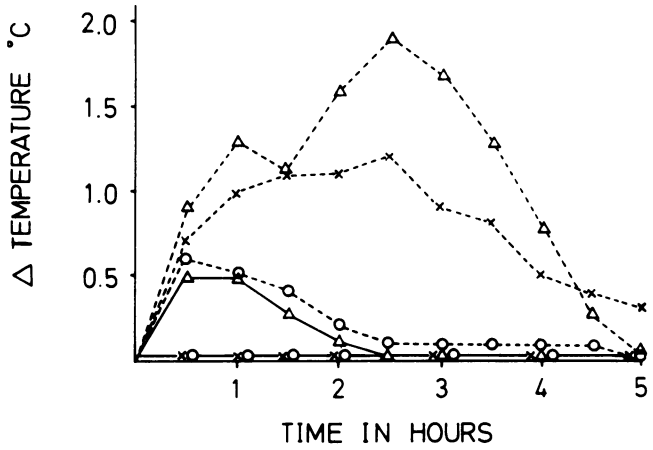


FIG. 3. Fever response to original and succinylated preparation 316. △, 50 μg/kg; x, 5 μg/kg; ○, 1 μg/kg. Solid line, original; dashed line, succinylated form.

temperature increases were about 500 times higher as compared with lipid A.

Table 1 summarizes the results obtained with the synthetic preparations. Typical pyrogenic responses with fever peaks 1 and 2.5 h after injection of 50 μg of sample per kg were observed with preparation 317 and succinylated preparations 302 and 316. Samples 316 and 321 and succinylated preparations 311 and 315 caused monophasic fever responses (50 μg/kg) similar to those observed with low doses of natural lipid A. None of the monosaccharide preparations expressed pyrogenicity.

Lethal toxicity of free original and succinylated lipid A. As shown in Table 2, lipid A expressed relatively low lethal activity in galactosamine-treated mice when injected intraperitoneally. This also applies to succinylated lipid A. On intravenous injection into galactosamine-pretreated mice, however, lipid A was highly active, and the same activity was found with the succinylated lipid A preparation. Thus, succinylation does not alter the toxic activity of lipid A, in agreement with previous results with original and succinylated *S. minnesota* Re 595 LPS (16).

TABLE 2. Lethal effects of free lipid A and succinylated free lipid A in galactosamine-sensitized C57BL/6 mice

Lipid A from *S. minnesota* Re595			
Original (orig) or succinylated (succ)	Route of injection[a]	Dose (μg/mouse)	No. dead/total no. (%)
orig	i.p.	1	0/8 (0)
orig	i.p.	10	3/8 (37.5)
succ	i.p.	1	1/8 (12.5)
succ	i.p.	10	5/8 (62.5)
orig	i.v.	0.001	0/10 (0)
orig	i.v.	0.01	7/15 (47)
orig	i.v.	0.1	10/10 (100)
succ	i.v.	0.001	0/8 (0)
succ	i.v.	0.01	4/8 (50)
succ	i.v.	0.1	8/8 (100)

[a] D-Galactosamine-hydrochloride (10 mg) was injected intraperitoneally (i.p.) in 0.5 ml of PBS. The samples to be tested were either mixed and injected with the galactosamine solution or injected separately by the intravenous (i.v.) route immediately after the administration of galactosamine.

TABLE 3. Lethal effects of synthetic lipid A analogs in galactosamine-sensitized C57BL/6 mice

Sample no.	Sample tested[a]	Solubility[b]	Dose (μg/mouse)	No. dead/total no.[c] (%)
301	orig	−	50	0/6 (0)
304	orig	−	50	0/3 (0)
303	orig	+	50	0/6 (0)
305	orig	+	50	0/6 (0)
302	succ	++	50	0/8 (0)
321	orig	+	50	0/6 (0)
316	orig	+	5	0/6 (0)
	orig	+	50	9/9 (100)
	succ	++	5	0/6 (0)
	succ	++	50	1/6 (17)
317	orig	++	1	0/6 (0)
	orig	++	5	4/12 (33)
	orig	++	50	6/6 (100)
311	succ	++	50	0/7 (0)
312	succ	++	50	0/4 (0)
314	succ	++	50	5/7 (71)
315	succ	++	1	0/4 (0)
	succ	++	10	3/8 (38)
	succ	++	50	6/6 (100)
318	orig	++	50	0/6 (0)
319	orig	++	50	0/6 (0)
313	orig	−	50	0/4 (0)
	succ	++	50	0/12 (0)

[a] orig, Original; succ, succinylated.
[b] ++, Solution; +, fine suspension; −, particles.
[c] Nonsensitized mice survived 100 μg of all preparations per mouse. No deaths were recorded in the animals receiving galactosamine only.

Lethal toxicity of synthetic preparations. Table 3 shows the results of toxicity tests with the synthetic lipid A analogs injected intravenously into galactosamine-sensitized mice. Preparations 316 and 317, which could be solubilized in water directly, exhibited 100% lethality with 50 μg per mouse, and 5 μg of preparation 317 per mouse still resulted in 33% mortality. Although preparation 316 was partly inactivated by succinylation, some originally insoluble preparations such as 314 and 315 became soluble and toxic after succinylation. With the most active preparations, 50 μg per mouse was needed for 100% lethality; compared to lipid A (0.1 μg) the activity of the analogs was about 500 times lower. As with lipid A under these conditions, the majority of the mice died between 5 and 9 h after injection of the preparations, providing an additional qualitative criterion for endotoxin-induced lethality in these animals (2). None of the synthetic preparations was toxic when administered intraperitoneally.

Anticomplement activity of free original and succinylated lipid A. Dose-response curves of the anticomplement activity of both lipid A and succinylated lipid A are shown in Fig. 4. The percent inhibition of hemolysis is plotted against the amounts of the test samples. Fifty percent inhibition of lysis was achieved with 5 μg of lipid A and 25 μg of succinylated lipid A. The anticomplement activity of lipid A is significantly decreased by succinylation.

Anticomplement activity of synthetic lipid A analogs. The anticomplement activities of the synthetic preparations were tested with maximal doses up to 100 μg. Dose-response curves of some of the active preparations are shown in Fig. 4. Succinylated preparation 301 was one of the most active ones, and the inhibition curve is very similar to that of succinylated lipid A. Table 4 summarizes the results. Recorded are the doses which caused 50% inhibition of lysis. Preparation 301, which contains only tetradecanoic acid residues and no phosphate, was insoluble in water and inactive in the original form. After succinylation it exhibited strong anticomplement activity (50% inhibition with 28 μg). Also, the other preparations belonging to the first group in Table 4 and containing tetradecanoic acid as the only type of acyl residues exhibited relatively strong anticomplement activity. The monosaccharide preparations were inactive.

***Limulus* gelation assay.** Bacterial lipid A, which was used as a reference, was positive in this test with a minimal dose of 2×10^{-6} μg/ml. None of the synthetic preparations tested (301, 302, 303, 304, 305, 313, 314, 316, 317, 318, and 321) exhibited gelation activity in doses of up to 0.4 μg/ml, i.e., 2×10^5 times more than the minimum effective dose of the natural products.

DISCUSSION

In the course of the chemical synthesis of lipid A, approached in several laboratories, a number of synthetic lipid A analogs became available which structurally resemble bacterial lipid A. We have undertaken an investigation of the

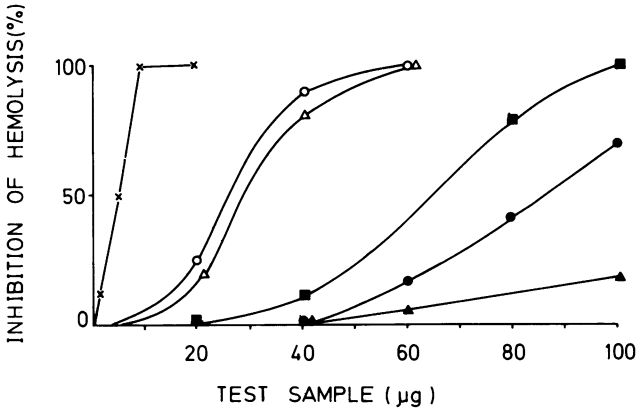


FIG. 4. Anticomplement activity of free lipid A from *S. minnesota* Re 595 and some synthetic lipid A analogs. x, Free lipid A; ○, its succinylated form; ■, synthetic preparation 304; ▲, synthetic preparation 316; ●, synthetic preparation 317; △, succinylated 301.

TABLE 4. Anticomplement activity of synthetic lipid A analogs

Sample no.	Dose for 50% inhibition (μg)	
	Original form	Succinylated form
Lipid A (*S. minnesota* Re 595)	5	25
301	>100	28
304	65	58
303	30	
305	41	
302	>100	65
321	>100	
316	>100	
317	82	
311	>100	85
312	>100	>100
314	>100	>100
315	>100	>100
318	>100	
319	>100	
313	>100	>100

biological activities of synthetic preparations to identify structure/activity relationships. In the present study the preparations were tested for four typical endotoxic activities, i.e., pyrogenicity, lethal toxicity, anticomplement activity, and induction of gelation of *Limulus* amoebocyte lysate. Bacterial free lipid A served as a reference. Some preparations, especially those containing phosphate groups, could be solubilized in water after conversion into the triethylammonium form. Most of the preparations, however, could not be solubilized directly. Since it had been shown previously with Re LPS (16) that the introduction of carboxyl groups increased solubility in water without altering a number of biological activities, some of the synthetic preparations were succinylated. It was confirmed again in this study that such a modification did not change the toxic activity and pyrogenicity of natural lipid A. A certain decrease regarding the anticomplement activity was, however, observed. Strong pyrogenic responses with biphasic fever curves (maximal temperature increase, 1.1 to 1.9°C) were obtained with 50 μg/kg doses of original preparation 317 and succinylated preparations 302 and 316. Compared to free lipid A, however, the activities were low: to obtain a comparable febrile response, 0.05 to 0.1 μg of free lipid A per kg was sufficient. A number of preparations (original 316 and 321 and succinylated 311 and 315) exhibited moderate pyrogenicity, expressing a monophasic fever curve with a temperature increase of 0.6°C (50 μg/kg).

We independently succinylated three different batches of preparation 302, two of which had been synthesized by different routes. All three succinylated preparations exhibited equal pyrogenicity, proving the reproducibility of activation by succinylation. On the other hand, a number of preparations did not exhibit pyrogenicity in the succinylated form (Table 1). This and the fact that the succinylated preparations were inactive in the *Limulus* test and nontoxic show that their activity as pyrogens is not due to contamination introduced into the preparations during and after the succinylation step.

Treatment of mice with D-galactosamine has been shown to increase their sensitivity to the lethal effect of LPS and lipid A by a factor of several thousand. As shown in Table 3, original preparations 316 and 317 and succinylated preparations 314 and 315 exhibited lethal toxicity under these conditions. Compared to bacterial lipid A, however, the activities of these preparations were much lower, as was also observed for pyrogenicity. For the same effect, about 500 times more of the synthetic preparations was required than of lipid A.

In the anticomplement test, original preparations 303, 304, 305, and 317 and succinylated preparations 301, 302, 304, and 311 were active. Preparation 301, which was quite insoluble in water, exhibited after succinylation almost the same activity as succinylated lipid A. The other preparations were less active.

None of the preparations was found to exhibit *Limulus* amoebocyte gelation activity when tested in doses of up to 0.4 μg/ml, whereas lipid A was still active at 2×10^{-6} μg/ml. These results exclude the possibility of an endotoxin contamination in the synthetic preparations.

The present results do not yet allow a clear identification of structural requirements for the activities, but some indications can be given. None of the monosaccharide derivatives exhibited activity. It is obvious that the preparations which are devoid of 3-hydroxytetradecanoic acid are inactive in both in vivo tests, pyrogenicity and lethal toxicity. It is, however, clear that pyrogenicity and lethal toxicity are not coupled. For instance, succinylated preparation 302 was found to be pyrogenic but not lethal, whereas succinylated preparations 314 and 315 were lethal but of no or low pyrogenicity. Furthermore, original preparation 316 was toxic and of low pyrogenicity, whereas its succinylated form was pyrogenic and of low toxicity. A similar separation of activities has also been observed with the lipid A precursor I molecule, which contains the lipid A backbone substituted by four 3-hydroxytetradecanoyl residues and which is toxic but of weak pyrogenicity (13). Phosphate groups at positions 1 and 4′, as well as hydroxyacyl and acyloxyacyl residues, are factors determining lethal toxicity. Regarding phosphate groups it is not easy to differentiate between their direct influence on the activity and their possible indirect influence through changes of physical properties, such as the solubility of the preparations. Different structural factors seem to be required also for anticomplement activity. For instance, all preparations containing tetradecanoic acid as the only fatty acid type (preparations 301, 303, 304, and 305) exhibited strong anticomplement activity independent of the phosphate substitution. These preparations did not exhibit pyrogenicity or lethal toxicity. On the other hand, pyrogenic and toxic preparations did not always exhibit comparable anticomplement activity.

The results indicate that all activities tested in our laboratory, pyrogenicity, lethal toxicity, anticomplement activity, and *Limulus* amoebocyte lysate gelation activity, are separable. This is also true regarding the results of Yasuda et al. (21) and Kotani et al. (11), who emphasized the importance of phosphate groups for adjuvanticity. Previous results with lipid A's derived from different bacterial families and differing in structure (1, 13, 18, 19) also have indicated that individual biological activities are separable. It seems that the presence of one single constituent is not decisive for endotoxic activities, but rather that several factors contribute to activity.

In this study, the most active synthetic preparations were shown to be less active than bacterial free lipid A. However, it must be kept in mind that so far none of the structures of the synthetic preparations is identical with that of bacterial lipid A, whose structural details, i.e, the position of *O*-acyl groups, have been identified only very recently (Rietschel et al., in press), after the lipid A analogs had been synthesized.

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