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Interaction of Lipopolysaccharides and Lipid A With Complement in Rats and Its Relation to Endotoxicity

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Uniform salt forms of endotoxic lipopolysaccharides (LPS) and free lipid A showing distinct differences in their anticomplementary activity, as well as a nontoxic LPS, are used in a new approach of studying the role of complement in endotoxin shock. The use of these defined LPS forms led to the identification of two timely, distinct depressions in complement hemolytic activity after administration of endotoxin in rats. An early depression occurred within 10 min after injection, and a late one developed more gradually, with lowest values at 6 to 9 h. The early depression represents a direct interaction of LPS with complement. It was obtained by toxic and nontoxic preparations that exhibit a high molecular weight and anticomplementary activity in vitro. The early depression was not related to the toxic properties of the LPS. The late depression was obtained only with endotoxically active LPS in lethal and 100-fold-lower concentrations, regardless of their molecular weight and of their in vitro anticomplementary activity.

The role of the complement system in endotoxin shock has been investigated ever since Spink and Vick (30) found that shock caused by lipopolysaccharides (LPS) required a heat-labile factor. It was repeatedly shown that injection of toxic amounts of LPS produces a rapid fall of complement levels (13, 25, 29). In vitro, many LPS exhibit anticomplementary activity that is embedded in the lipid A part of the molecule (20). The interaction proceeds via the alternate pathway, involving the terminal complement components C3 to C9 (12), and was shown to lead to the development of biologically active split products (14, 19, 28); however, their significance in vivo remained unclear.

In vivo investigations in animals depleted of late complement components by cobra venom factor showed that the role of complement in endotoxin shock was protective, damaging (3, 4), or without effect (2), according to the conditions used. Animals genetically deficient in C4, C5, and C6 showed a somewhat higher susceptibility to the lethal effects of endotoxin than did conventional animals, which supports the view that complement was not playing a damaging role in endotoxin shock (15, 16, 22). On the other hand, all chemical modifications of LPS that led to extensive loss of toxicity also abolished anticomplementary activity (11).

Very recently it was shown that the expression of in vitro anticomplementary activity depends on the physical state of LPS and requires a high molecular weight (apparent molecular weight due to aggregation) (7). The high aggregation usually shown by LPS could be altered by electrodialysis and neutralization with different bases. This yielded LPS in uniform salts exhibiting large but constant differences in their molecular weight (6). The chemical integrity of the LPS remained thereby intact.

Two forms representative of high and low molecular weights are the sodium and triethylamine salts, respectively, the former being highly anticomplementary, and the latter being virtually inactive. Both forms were highly toxic for adrenalectomized mice and pyrogenic in rabbits (5). The availability of endotoxic LPS in anticomplementary and non-anticomplementary forms offers further possibilities for studying the role of complement in endotoxic reactions.

In the present study, the interaction of the triethylamine and sodium forms of *Salmonella abortus-equi* smooth (S) form, *S. minnesota* R595, and *Salmonella* free lipid A with rat complement was studied in vivo and in vitro and compared to their lethal toxicity.

Very recently a number of LPS derived from the family of photosynthetic bacteria have been isolated (31) that differ in the basic structure of their lipid A component, compared to enterobacteriaceae (27). One such preparation, the LPS of *Rhodopseudomonas viridis* F, was shown previously (10) to interact with complement in vitro, but to express no lethal toxicity. Studying the in vivo interaction of this nontoxic

LPS with rat complement is a new approach in examining the role of complement in endotoxicity.

MATERIALS AND METHODS

LPS and free lipid A. The *S. abortus-equi* S-form LPS was isolated from bacteria by the phenol-water method (32). It was freed of glycan and rough (R) form-like material as described previously (6). The *S. minnesota* R595 (Re) LPS was isolated from bacteria by the phenol-chloroform-petroleum ether procedure (8). Both LPS were freed from metal cations and basic amines by extensive electrodialysis, as described previously, whereby the acid form of the LPS was obtained. They were converted to the sodium and triethylamine forms by neutralizing with the respective base and lyophilized. They were stored over calcium chloride at 4°C.

Soluble free lipid A in the sodium and triethylamine forms was prepared from electrodialyzed *S. minnesota* R345 (Rb) (21) LPS as described previously (6).

The *R. viridis* F LPS prepared by the phenol-water method was a gift from J. Weckesser. It was deionized by electrodialysis and converted to the sodium form by neutralizing with sodium hydroxide.

Sedimentation coefficient determinations of LPS and free lipid A were carried out in a Beckmann analytical ultracentrifuge as described earlier (6).

Animals. Female AS 2 rats were bred under specific pathogen-free conditions at our institute. They were used at 12 weeks of age. For injections, solutions of each LPS and lipid A were prepared in pyrogen-free distilled water. When necessary, the solutions were adjusted to pH 7.0 by using the corresponding base for each salt form. In all cases the LPS was administered intravenously in the lateral tail vein in a volume of 0.6 ml. Control animals received 0.6 ml of distilled water. Blood for complement titrations was collected under ether anesthesia from the tail immediately before and at different times up to 48 h after injection. The blood was allowed to clot in ice for 3 h and was centrifuged at 4°C, and the serum was separated and frozen at −75°C until use. A maximum of six bleedings, each not exceeding 0.3 ml, was carried out on any single animal. All animals used showed no detectable anti-*S. abortus-equi*, -*S. minnesota* R595, -*R. viridis*— F, or -lipid A antibodies in their sera, as measured by the passive hemolysis test (24).

Complement titration. The serum samples from individual animals were tested simultaneously. From each serum different amounts (2 to 50 μl) were placed in plastic tubes in a total volume of 50 μl. Sheep erythrocytes (5×10^8) sensitized with amboceptor in Veronal buffer (1.5 ml) were added and incubated at 37°C for 1 h. They were centrifuged in the cold, hemolysis in the supernatants was measured spectrophotometrically at 546 nm, and the 50% hemolytic complement (CH_{50}) units were estimated (17). In all animals the starting hemolytic activity of the serum varied between 110 and 140 CH_{50} units per ml.

Assay of in vitro anticomplementary activity. Fresh pooled rat serum (100 μl) was incubated with increasing amounts (3 to 50 μg) of LPS and lipid A in distilled water (5 μl) at 37°C for 1 h. Portions (12 μl) were transferred into tubes containing 5×10^8 sensitized sheep erythrocytes in 1.5 ml of Veronal buffer. They were incubated at 37°C for 1 h and centrifuged, and hemolysis in the supernatant was measured spectrophotometrically at 546 nm. Under these conditions the complement control (12 μl of serum without LPS) showed a 70% lysis of the erythrocytes.

The fresh rat serum used contained no detectable anti-*S. abortus-equi*, -*S. minnesota* R595, -*R. viridis* F, or -lipid A antibodies, as measured by the passive hemolysis test (24).

Lethal toxicity of LPS and lipid A in rats. Different concentrations (1 to 12 mg) of each preparation dissolved in distilled water (0.6 ml) were injected intravenously in the lateral tail vein of AS 2 rats under ether anesthesia. For each concentration, 10 animals were used. The 50% lethal dose (LD_{50}) was calculated by the method of Reed and Muench (26).

RESULTS

In vitro interaction of LPS and lipid A in sodium and triethylamine forms with rat complement. Increasing amounts of the sodium and triethylamine forms of the LPS of *S. abortus-equi*, *S. minnesota* R595, *R. viridis*, and lipid A were incubated with 100 μl of fresh rat serum, and the resulting loss of hemolytic activity was measured as described above (Fig. 1). The sodium form of both *Salmonella* LPS exhibited high anticomplementary activity. A 50% loss of hemolytic activity was obtained with 16 μg of the S form and 7 μg of the R form. In contrast, the low-molecular-weight triethylamine form of *S. abortus-equi* was completely inactive, and that of *S. minnesota* R595 (50-μg concentration) showed a mere 17% inhibition of hemolytic activity. The sodium form of *R. viridis* LPS exhibited high anticomplementary activity, showing a 50% loss of hemolytic activity with 4 μg. With free lipid A, both the sodium and the triethylamine forms were anticomplementary, showing a 50% complement inactivation with 23 and 29 μg, respectively.

In a previous study, the sedimentation coefficients measurement in an analytical ultracentrifuge revealed that the molecular weight (apparent molecular weight due to aggregation) of the sodium salt of LPS was considerably higher than that of the triethylamine salt (6). This is also true for the *Salmonella* LPS used in the present study. The sedimentation coefficients of the triethylamine form of both the S- and the R-form LPS and of free lipid A are low, whereas those of the sodium form are considerably higher (Table 1). A high sedimentation coefficient was also found for the *R. viridis* sodium form.

Lethal toxicity of LPS and lipid A. The LD_{50} values of the LPS and lipid A preparations, tested as described above, are presented in Table 2.

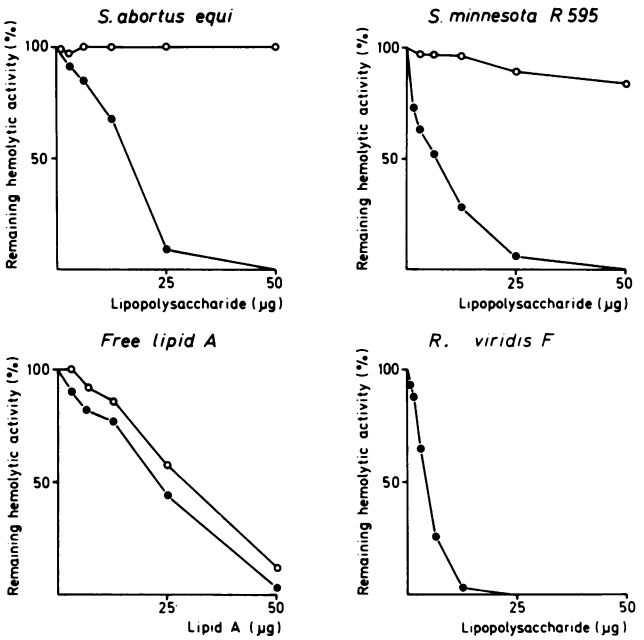

FIG. 1. *In vitro anticomplementary activity of LPS and lipid A in the sodium and triethylamine forms. Increasing amounts of each preparation were incubated with 100 μl of rat complement, and loss of hemolytic activity was measured. Symbols: ●, sodium forms; ○, triethylamine forms.*

TABLE 1. *Sedimentation coefficients of preparations*

Prepn	*s* value (S, 10^{-13} s)
S. abortus-equi	
Sodium	80
Triethylamine	4
S. minnesota R595	
Sodium	40
Triethylamine	2.5
Salmonella free lipid A	
Sodium	82
Triethylamine	2.5
R. viridis F	
Sodium	400

TABLE 2. *Lethal toxicity in rats*

Prepn	LD_{50} (mg)
S. abortus-equi	
Sodium	5.3
Triethylamine	6.2
S. minnesota R595	
Sodium	4.3
Triethylamine	6.4
Salmonella free lipid A	
Sodium	3.2
Triethylamine	10
R. viridis F	
Sodium	>20[a]

[a] With 20 mg no deaths were recorded.

The sodium form of the *Salmonella* LPS and lipid A was more toxic than the triethylamine form. In S-form LPS, the differences in the toxicity of sodium (LD_{50} = 5.3 mg) and triethylamine (LD_{50} = 6.2 mg) salts are marginal. They were more pronounced in sodium (LD_{50} = 4.2 mg) and triethylamine (LD_{50} = 6.4 mg) salts of Re LPS and even more in free lipid A, where the sodium form (LD_{50} = 3.2 mg) shows the highest and the triethylamine form (LD_{50} = 10 mg) shows the lowest lethal toxicity.

The LD_{50} of the *R. viridis* preparation in rats could not be estimated due to the low toxicity of the preparation. With 20 mg of LPS injected in two animals, no deaths were recorded and no visible signs of illness were observed. The absence of toxicity in this LPS measured in more sensitive tests has been described earlier (10).

In vivo interaction of toxic LPS and lipid A in the sodium and triethylamine form with complement. A 6-mg amount of each salt form of *S. abortus-equi*, *S. minnesota* R595, and free lipid A in 0.6 ml of distilled water was injected intravenously in groups of rats. Animals receiving 0.6 ml of distilled water adjusted to pH 7.0 with either NaOH or triethylamine served as

controls. Before injection and at various times up to 48 h thereafter, blood (ca. 0.3 ml) was removed and serum complement hemolytic activity was measured as described above.

The hemolytic activity of the sera during this period is shown in Fig. 2. Each point on the curves represents the mean value from at least 10 animals in LPS and at least 6 animals in free lipid A. The two LPS show a similar pattern of complement inactivation when present in the same salt form. However, the two salt forms show distinct differences between them. In the sodium form, both LPS induced an almost immediate fall in complement values, the hemolytic activity being reduced to about 20% of the preinjection levels. Apart from a slight increase at 1 h, the complement activity remained low up to 9 h. In surviving animals, hemolytic activity then rose, up to 48 h, with the R-form preparation, the recovery being somewhat slower than with the S form.

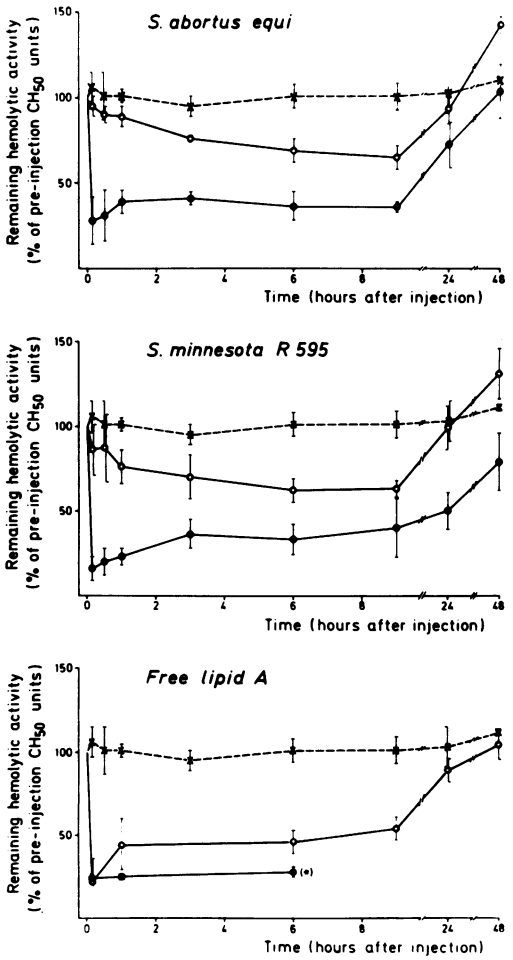

FIG. 2. *In vivo anticomplementary activity of LPS and free lipid A in the sodium and triethylamine forms. Six milligrams from each preparation was injected intravenously in AS 2 rats. Before injection and at different times thereafter, the serum hemolytic activity was measured. Symbols: ●, sodium forms; ○, triethylamine forms; X, control rats receiving 0.6 ml of pyrogen-free distilled water, 1 standard deviation.*

A different pattern in the changes of complement hemolytic activity was found in the animals receiving 6 mg of the triethylamine form of the two LPS. Here, the early fall obtained with the sodium-form preparations was absent, and only a slow, gradual fall with lowest values (about 60% of original activity) measured at 6 to 9 h after injection was present. After 24 h the hemolytic activity in surviving animals was nearly restored, as well as after 48 h above preinjection levels.

In contrast to the two LPS, lipid A in both the sodium and triethylamine forms induced the early drop in complement activity (Fig. 2). In both cases, about 20% of the starting hemolytic activity was detected 10 min postinjection. In the sodium form, all animals retained this low complement activity up to 6 h postinjection. Beyond this, time measurements were not possible, as all animals in this group died in shock between 8 and 9 h postinjection. With the triethylamine form of lipid A, the low values of complement activity persisted up to 9 h postinjection. The overall pattern of complement hemolytic activity over a 48-h period is very similar to that obtained with the sodium form of the two LPS.

In the control group of animals receiving 0.6 ml of distilled water, no significant changes in complement levels were measured. The hemolytic activity remained constant throughout the 48 h postinjection.

The 6-mg quantities of the two LPS and lipid A represent a toxic dose. All animals receiving this amount showed acute signs of toxicity (rough fur, immobility, hemorrhage in the eyes and nose, refraining from eating and drinking), and many of them died.

In addition to the 6-mg quantities, lower nonlethal amounts of the sodium and triethylamine forms of the two LPS were tested. Injections of 600 and 60 μg led in all cases to a slow, gradual fall in hemolytic activity, with the lowest values obtained again after 6 h (Table 3). An early fall was detectable only with the 600-μg concentration of the sodium form of the R-form LPS. The 600- and 60-μg concentrations showed in all cases no visible toxic effects, and the signs of illness described above were not observed. With the 6-μg concentration (not shown in Table 2), no changes in complement were observed, and the pattern of hemolytic activity was indistinguishable from that of the controls.

TABLE 3. *Effect on complement of different concentrations of LPS preparations in rats*

LPS (mg)	% of original CH_{50}			
	10 min[a]	1 h[a]	6 h[a]	24 h[a]
S. abortus-equi				
Sodium				
6.0	28 (±14)[b]	39 (7)	36 (8)	73 (14)
0.6	97 (10)	86 (4)	76 (5)	123 (8)
0.06	97 (11)	85 (4)	73 (9)	117 (7)
Triethylamine				
6.0	95 (6)	89 (6)	69 (7)	94 (9)
0.6	98 (3)	89 (5)	75 (5)	117 (13)
0.06	99 (3)	92 (3)	72 (2)	121 (16)
S. minnesota R595				
Sodium				
6.0	16 (7)	23 (5)	33 (9)	50 (11)
0.6	59 (10)	58 (10)	58 (6)	121 (16)
0.06	96 (3)	93 (5)	75 (3)	110 (3)
Triethylamine				
6.0	86 (15)	76 (10)	61 (7)	99 (14)
0.6	97 (4)	87 (7)	77 (5)	103 (3)
0.06	105 (3)	96 (4)	82 (7)	129 (4)
Control				
0.6 ml of distilled water	106 (9)	101 (4)	101 (7)	103 (12)

[a] Time after LPS injection.

[b] Values in parentheses represent standard deviation.

In vivo anticomplementary activity of the nontoxic *R. viridis* F LPS. The pattern of complement inactivation obtained with 6 and 0.6 mg of the sodium form of *R. viridis* is shown in Fig. 3. For each concentration, groups of three animals each were used. In contrast to the toxic preparations, the *R. viridis* LPS induced only an early, short-lived depression in complement activity. With 6 mg of LPS, complement titers measured 10 min postinjection were reduced to 13% of preinjection values. Thereafter, complement activity rose continuously in all animals, and preinjection values were reached within 24 h. The long-lived low hemolytic titer obtained with the toxic LPS was absent. A similar pattern of complement inactivation and recovery was also seen with 0.6 mg of LPS; here, however, the loss of hemolytic activity was lower than with 6 mg. With 0.06 mg of LPS, changes in complement compared to the control were seen.

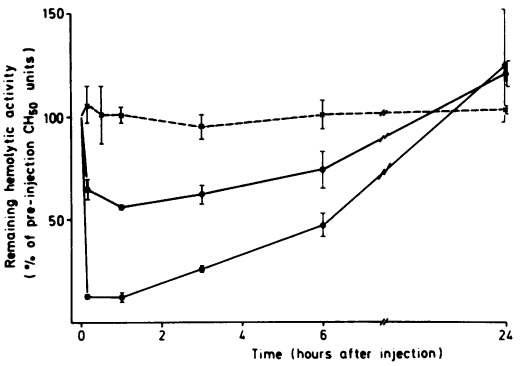

FIG. 3. *In vivo anticomplementary activity of R. viridis F. LPS in the sodium form. Two different concentrations were injected intravenously in AS 2 rats. Before and at different times thereafter, the serum hemolytic activity was measured. Symbols: ●, anticomplementary activity with 6 mg; ○, anticomplementary activity with 0.6 mg; X, controls rats receiving 0.6 mg of distilled water, 1 standard deviation.*

DISCUSSION

The present results show that LPS, regardless of their toxicity, interact with rat complement in vitro only when they are present in a state of high aggregation. These data agree with those of a previous study with guinea pig complement (7).

In vitro both the *S. abortus-equi* S- and the *S. minnesota* Re-form LPS were strongly anticomplementary in the high-molecular-weight sodium form. In the low-molecular-weight triethylamine form, anticomplementary activity was absent from the S form and only weakly expressed by the R form. High anticomplementary activity was also shown by the *R. viridis* F LPS. In lipid A, both the sodium and triethylamine salt forms were anticomplementary. The anticomplementary activity of the triethylamine form of lipid A, despite its low molecular weight (5*s*), is not contradictory to the concept of a high molecular weight being necessary for the expression of anticomplementary activity. It is explained by the more expressed property of lipid A to reaggregate through binding of diva-

lent cations present in serum into a high-molecular-weight anticomplementary form. R-form LPS in the triethylamine form may also interact with complement due to reaggregation as shown in a previous study (7). However, when exhaustively electrodialyzed, as in the Re preparation used in the present study, the triethylamine form is stable and shows no interaction with complement. The amounts of Mg^{2+} and Ca^{2+} in the serum seem to represent borderline concentrations for these lipophilic LPS. Consequently, incomplete electrodialysis may result in preparations containing cations other than triethylamine in concentrations high enough to lower the threshold of aggregation, leading to anticomplementary forms. In contrast, the triethylamine salt of S-form LPS is stable in the presence of divalent cations and retains its low-molecular-weight inactive form (7).

The fresh rat serum used as source of complement contained no detectable antibodies to any of the preparations used. Therefore, antibody-mediated complement consumption was absent, and the complement inactivation observed was due to a direct interaction of the LPS and lipid A with complement.

The interaction of LPS with complement is known to proceed via the alternative pathway (5). However, evidence has been presented (1, 18, 23) that only S-form LPS activate the alternative pathway, whereas R-form LPS and lipid A act via the classical pathway. The present data show, therefore, that in the interaction of LPS with complement, a high molecular weight is a prerequisite for the activation of both pathways.

The results of the in vivo studies revealed that LPS may induce two timely, distinct depressions in complement activity. An early, rapid depression occurred within 10 min postinjection, and a delayed, more gradual one occurred with the lowest complement titers measured 6 to 9 h postinjection.

The following patterns were obtained (Table 4): the sodium form of the *Salmonella* LPS induced both the early and delayed depressions, the latter being best visible with 0.6 and 0.06 mg of LPS (Table 3). With the 6-mg concentration (Fig. 2), it is masked by the effect of the early depression and may not be clearly recognized as an independent activity. The triethylamine form of the *Salmonella* LPS induced only the delayed depression. The *Salmonella* lipid A in both salt forms induced the early depression with 6 mg. Lower concentrations that might have revealed the possible presence of a delayed depression were not tested. The nontoxic *R. viridis* F preparation exhibited only the early depression in complement activity (Table 4).

Thus, the early drop was induced by preparations that also exhibited in vitro anticomplementary activity. It was detectable only with high concentrations, i.e., with amounts equivalent to those showing detectable anticomplementary activity in vitro. This drop, therefore, is a result of a direct interaction of LPS with complement. The delayed drop was obtained only with toxic preparations and was independent of the salt form and of in vitro anticomplementary activity. It was induced to a comparable extent with lethal and 100-fold-lower amounts, indicating that it is not a direct effect of the LPS, but is caused indirectly through mechanisms generated in the course of endotoxicity. Thus, the two depressions in complement activity develop independently and are due to distinctly different mechanisms.

The AS 2 strain of rats is relatively resistant to endotoxin. It was chosen for the present investigation because it allows the interaction of large amounts of LPS with complement (early depression) to be detected and measured and thereby clearly differentiated from the indirect complement inactivation (delayed depression).

An analysis of lethal toxicity data allows some understanding of the biological significance of the two depressions. Although the early depression occurred within 10 min of injection, visible signs of illness (rough fur, immobility, etc.) occurred only after h 3; in the animals receiving the nontoxic *R. viridis* preparation, the signs of illness described above were never present. Considering the high extent of complement inactivation obtained, it would seem obligatory to conclude that in rats the direct activation of complement by LPS is not disastrous, per se, for these animals.

This obviously is also true for the complement consumption occurring during the delayed depression, where lethal and 100-fold-lower doses (in the absence of any visible signs of illness)

TABLE 4. *Toxic and anticomplementary properties of preparations*

Prepn	Toxic	In vitro interaction with complement	In vivo early depression	In vivo delayed depression
Salmonella LPS Sodium	+	+	+	+
Triethylamine	+	−	−	+
Salmonella free lipid A Triethylamine and sodium	+	+	+	ND[a]
R. viridis LPS Sodium	−	+	+	−

[a] ND, Not determined.

had a comparable effect on complement. Thus, the complement consumption itself is not directly responsible for toxicity of illness. Since, however, the delayed depression was obtained only with toxic preparations, it may simply signal the presence of a toxic stimulus, thus being the effect of toxicity rather than the cause.

Administration of LPS in experimental animals leads to the development of numerous biological activities, many of which are not necessarily harmful for the organism. Many of these are indirect endotoxin activities produced by endogenous mediators released along the chain of events that follow the interaction of endotoxin with primary targets. In the early complement depression demonstrated in the present study, complement may be seen as a primary target of endotoxin activity. In rats, however, this complement activation was not related to endotoxicity, and any resulting biological activities are not expected to be disastrous for these animals. The various preparations used in the present study may prove suitable vehicles for studying such activities and may thus help to identify the clone of endotoxin activities originating from the interaction of LPS with complement.

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