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LIPID A COMPONENT OF LIPOPOLYSACCHARIDES FROM *COXIELLA BURNETII*

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Summary. — Free lipid A from phase I and phase II *Coxiella burnetii* lipopolysaccharides was isolated and studied serologically. Antisera against lipid A from phase I and phase II *C. burnetii* cross-reacted with the lipid A preparations from both phases as well as with *Salmonella* lipid A.

Key words: Coxiella burnetii; lipid A

Introduction

Lipopolysaccharides (LPS) from *Coxiella burnetii* resemble those of *Enterobacteriaceae* in that they also consist of a heteropolysaccharide which is linked to lipid A (Schramek and Brezina, 1976, 1979). But the endotoxic activity (lethal toxicity in mice) of *C. burnetii* LPS appears to be lower compared to that of *Salmonella*. The present communication deals with the isolation and partial serological characterization of the lipid A components of *C. burnetii* phase I and II cells. The lethal activity of the lipid A components for mice was also studied.

Materials and Methods

C. burnetii strain Nine Mile phase I (5th yolk sac passage cloned in cell cultures, kindly supplied by Dr. R. A. Ormsbee, Rocky Mountain Laboratory, Hamilton, Montana, U.S.A.) and phase II (157th yolk sac passage, from the Institute of Virology, Bratislava) was propagated in chick embryo yolk sacs. The rickettsial cells were killed by formalin and purified as described by Schramek *et al.* (1978).

Preparation of the LPSs. Purified cells of *C. burnetii* phase I and phase II were washed twice with ethanol and ether. The dried cells were suspended in distilled water and mixed with an equal volume of 90 % phenol (Westphal *et al.*, 1952). The mixture was incubated for 10 min at 68 °C under mixing, cooled and centrifuged for 20 min at 4000 × g. The aqueous phase was separated. The rickettsial residue in the phenol phase was extracted once more with the same amount of distilled water. The pooled aqueous phases were dialyzed against distilled water, passed through a membrane filter of 300 nm porosity and concentrated by lyophilization to a small volume. After centrifugation at 105,000 × g for 4 hr, the transparent pellet obtained was dissolved in distilled water and the solution centrifuged again. The sediment was lyophilized; the yield of LPS was 35 and 12 mg per 1 g of dried rickettsial cells for phase I and phase II, respectively.

Preparation of anti-lipid A antisera. Antisera to lipid A of *C. burnetii* phase I and phase II were prepared by immunization of rabbits with acid-treated rickettsial cells. The cells were

washed with 1 % acetic acid, then suspended in 1 % acetic acid and heated at 100 °C for 2 hr. They were washed with distilled water, suspended in physiological saline and used for intravenous immunization according to the following scheme: on days 1, 4, 7 and 11, the rabbits were given 0.15, 0.3, 0.5 and 1 mg, respectively. The rabbits were bled on day 17. Antiserum to lipid A of *Salmonella minnesota* R 595 was prepared as described (Galanos *et al.*, 1971). All sera were absorbed with sheep red blood cells and inactivated at 56 °C for 30 min before use for serological determinations.

Serological tests. Anti-lipid A titres were determined by the passive haemolysis test (Galanos *et al.*, 1971). Sheep erythrocytes were sensitized with isolated free lipid A solubilized by the addition of triethylamine at pH 7.4. The test was performed in small haemaglutination plates. Antisera were serially diluted twofold with veronal buffer (pH 7.4): 50 µl of each dilution were mixed with 50 µl of lipid A-sensitized erythrocytes. Finally 25 µl complement (guinea-pig serum diluted 1 : 10 with veronal buffer) were added and the plate was gently agitated. After incubation at 37 °C for 1 hr, haemolysis was recorded. The passive haemolysis inhibition test was carried out as follows: 25 µl of inhibitor in 25 µl of veronal buffer (pH 7.4) and 25 µl of anti-lipid A antiserum containing 2-3 haemolytic units were incubated at 37 °C for 15 min. Then 50 µl of sheep erythrocytes sensitized with lipid A and 25 µl complement were added to the mixture. After incubation at 37 °C for 1 hr, haemolysis was recorded.

Lethal toxicity was tested in galactosamine-sensitized mice (Galanos *et al.*, 1979). Galactosamine (10 mg) and samples of LPS or lipid A were injected as a mixture intraperitoneally into a group of 10 mice (C57Bl/6). Deaths occurring up to 24 hr after injection were recorded and the LD_{50} calculated by method of Reed and Muench.

Results

Free lipid A was prepared from LPSs of *C. burnetii* phase I or phase II by hydrolysis with 1 % acetic acid at 100 °C until precipitation of lipid A was complete. Since with phase I LPS liberation of lipid A was accomplished only after 2-3 hr, a more drastic treatment with 0.1 M hydrochloric acid was also applied. Free lipid A was separated from the reaction mixture by centrifugation (2000 × g, 10 min), washed several times with distilled water and dried in vacuo. Lipid A suspended in water was solubilized by the addition of triethylamine to a pH of 7.4.

Increasing amounts of solubilized free lipid A preprarations from phase I and II of *C. burnetii* were used for sensitization of sheep erythrocytes for the passive haemolysis test. Erythrocytes sensitized with lipid A from *Salmonella minnesota* were prepared similarly. In the actual test we used

Table 1. Serological cross-reactions between various lipids A and anti-lipid A antisera

Antisera to	Passive haemolysis titres with erythrocytes sensitized with lipid A isolated from		
	C. burnetii		*S. minnesota*
	Phase I	Phase II	
C. burnetii phase I lipid A	265	512	512
C. burnetii phase II lipid A	128	128	128
S. minnesota R 595 lipid A	1024	2048	2048
C. burnetii phase I	< 4	4	8
C. burnetii phase II	< 4	4	4
Normal serum	< 4	< 4	< 4

The titres are given as reciprocals of the highest serum dilution.

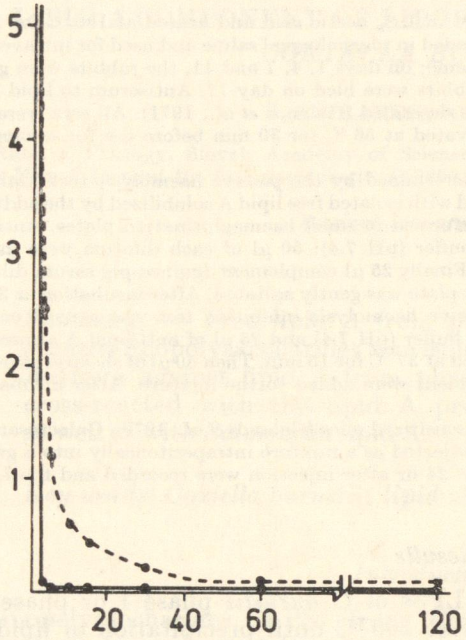

Fig. 1.

Kinetics of cleavage of lipid A during acid hydrolysis of LPS from phase I (– – –) or phase II (———) *C. burnetii*
Abscissa: time in min; ordinate: minimal dose (μg) inhibiting the passive haemolysis test

a concentration of lipid A which resulted in optimal sensitization. Table 1 shows that the 3 antisera exhibited lipid A titres not only in the homologous systems, but that they cross-reacted equally well in the respective heterologous systems.

The degree of cross-reactivity was determined by inhibition of passive haemolysis of the homologous systems by the different lipid A preparations.

The results (Table 2) showed that all lipid A preparations acted as potent inhibitors of both the homologous and the heterologous systems. LPS was not inhibitory.

As expected, the antisera reacted only with free lipid A, but not with the LPSs, containing bound lipid A. The antisera, therefore, contained no anti-0 specificity.

The kinetics of the liberation during acid hydrolysis of lipid A from LPS of either phase of *C. burnetii* were studied. Samples obtained at different periods of acid hydrolysis (1 % acitic acid, 100 °C) were neutralized by the addition of triethylamine and tested for their capacity of inhibiting passive haemolysis of the heterologous system *S. minnesota* lipid — *S. minnesota* anti-lipid A antiserum. The inhibitory activity of the samples increased with time of hydrolysis (Fig. 1). After 5 min for phase II and 50 min for phase I, maximal inhibition of LPS was obtained and this remained constant. Since only free lipid A reacted in this test, these data indicate that the liberation of lipid A was completed after 5 and 50 min, respectively, under the conditions used.

Table 2. Passive haemolysis inhibition of the system lipid A — antiserum to lipid A

Inhibitor	The system lipid A — antilipid A antiserum		
	C. burnetii		*S. minnesota*
	Phase I	Phase II	
C. burnetii phase I lipid A	0.08	0.04	0.04
C. burnetii phase II lipid A	0.04	0.02	0.02
S. minnesota R 595 lipid A	0.08	0.04	0.02
C. burnetii phase I LPS	> 5	> 5	> 5
C. burnetii phase II LPS	> 5	> 5	> 5

The figures in the Table refer to the lowest amount (expressed in μg) of inhibitor causing inhibition of passive haemolysis.

Treatment of mice with galactosamine increases lethal susceptibility towards LPS by a factor of about 10^4 (Galanos *et al.*, 1979). We tested LPS and lipid A from *C. burnetii* in comparison to *S. minnesota* LPS and lipid A. The results are summarized in Tab. 3. It is clear that the preparations from *C. burnetii* were far less toxic than those from *S. minnesota*.

Table 3. Comparison of the lethal effect of C. burnetii lipid A and the parent lipopolysaccharides in galactosamine-treated mice

Substance tested	Lethality for mice LD_{50} (μg)
C. burnetii phase II lipid A	5.2
S. abortus equi lipid A	< 0.001
C. burnetii phase I LPS	0.5
C. burnetii phase II LPS	3.5
S. abortus equi LPAS	< 0.001

Discussion

Lipid A of *Enterobacteriaceae* represents the endotoxic principle of lipopolysaccharides (endotoxins). It is immunogenic under certain conditions. Due to structural similarities, lipids A from different enterobacterial genera exhibit cross-reactions (Galanos *et al.*, 1977).

The fact that LPS from *C. burnetii* phase I and phase II are possess relatively low lethal toxicity for mice has prompted an investigation of the respective lipid A components.

The isolation of LPS by the phenol-chloroform-petroleum ether method from phase II *C. burnetii* has been described (Schramek and Brezina, 1979) but the method used here results in higher yields. It was found that lipid A was liberated from LPS after 5 to 50 min, as determined serologically, but more drastic methods had to be applied (especially in the case of phase I LPS) for precipitating free lipid A. This reminds of enterobacterial S and R form LPS; lipid A is faster liberated and precipitated from R-form LPS.

Lipid A of both phases was found to be immunogenic. Moreover the antisera obtained showed complete cross-reaction with *Salmonella* lipid A, indicating common immunodominant structures in the lipid A molecules. However LD_{50} determinations in galactosamine-sensitized mice demonstrated that phase I and II LPS and lipid A exhibit low toxicity, showing that structural dissimilarities must exist in the lipids A from *Salmonella* and *C. burnetii*.

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