IMPROVED METHOD OF ISOLATION OF *COXIELLA BURNETII* PROTEINS

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Summary. – An improved method of isolation of *Coxiella burnetii* proteins was developed. It consits of a combination of detergent (sodium dodecyl sulphate (SDS) or sodium deoxycholate (DOC)) and hot phenol treatments. The resulting phenol phase (PP) contained either lipopolysaccharide-(LPS) free proteins (DOC extraction) or proteins contaminated with LPS (SDS extraction), while the water phase (WP) contained LPS. Isolated *C. burnetii* proteins induced in mice and rabbits antibodies reacting in immunoblot analysis with both phase I and II *C. burnetii* corpuscles. A rabbit serum against *C. burnetii* prepared by DOC-phenol extraction did not react with purified phase I *C. burnetii* LPS in immunoblot analysis.

Key words: *Coxiella burnetii*; proteins; lipopolysaccharide; purification; sodium dodecyl sulphate; sodium deoxycholate; phenol; antibodies; immunoblot analysis

Introduction

Bacterial cell wall mediates the resistance of bacteria to host defense mechanisms, the acquisition of essential nutrients, and the adhesion to host tissues. It is outer membrane proteins that are involved in all these processes. When Gram-negative bacteria were exposed to detergents in extracting the outer membrane proteins, a significant amount of LPS accompanied these extracts (Ingalls *et al.*, 1995). The purification of proteins by various mostly chromatographic methods is difficult and time-consuming procedure (Benz and Bauer, 1988; Caldwell *et al.*, 1981).

The LPS-protein complex forms an important structure of the outer membrane of *C. burnetii*, but its role in the pathogenicity and immunogenicity of this obligatory intracellular parasite is not yet known. To disclose the role of proteins and LPS in the host-parasite interaction, their isolation in pure form is an important prerequisite. Various detergents and trichloroacetic acid (TCA) extract from *C. burnetii* the outer membrane protein-LPS complex (Lukáčová *et al.*, 1989; Vavreková *et al.*, 1992). This complex is very stable and a simple chromatografic separation of proteins from LPS was not successful (Lukáčová *et al.*, 1994). The purpose of this study was to find an isolation method for *C. burnetii* outer membrane proteins free from LPS without use of repeated chromatographic procedures. The protein-LPS complex obtained after the SDS or DOC extraction of *C. burnetii* cells according to a modified method of Caldwell *et al.* (1981) was dissociated by classical phenol method of LPS isolation (Westphal and Jann, 1965). Whereas the water phase (WP) contained LPS, the phenol phase (PP) consisted predominantly of proteins.

Abbreviations: CBB = Coomassie Brilliant Blue R 250; DOC = sodium deoxycholate; i.p. = intraperitoneally; i.v. = intravenously; LPS - lipopolysaccharide; PAGE = polyacrylamide gel electrophoresis; PP = phenol phase; TCA = trichloracetic acid; SDS = sodium dodecyl sulphate; WP = water phase

Materials and Methods

C. burnetii strain Nine Mile serologically in phase I (the third yolk sac passage) was propagated in chick embryo yolk sacs. Phenol-killed cells were purified by differential centrifugation and ether extraction (Ormsbee, 1962).

Detergent and phenol extractions. Suspensions (2 mg/ml) of *C. burnetii* corpuscles were shaken with 0.1% SDS (Pierce) or 1% DOC (Fluka) in solution of 0.1 mol/l Tris and 8 mmol/l EDTA,

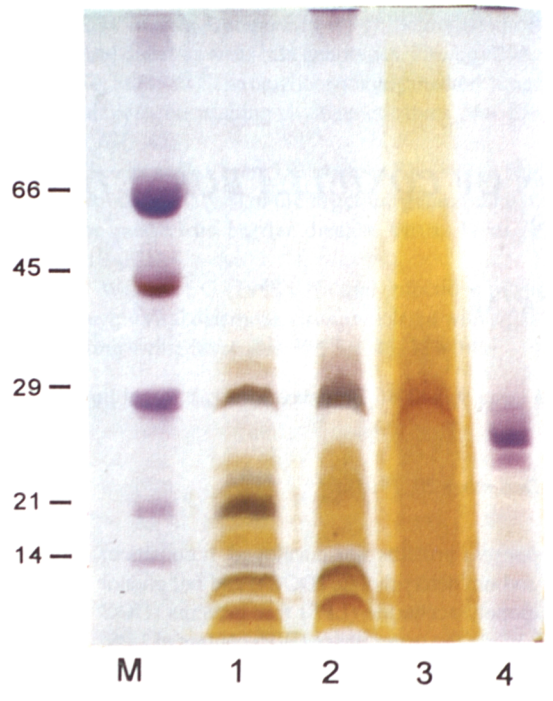

Fig. 1
SDS-PAGE of proteins and LPS extracted from *C. burnetii* with SDS and phenol
Whole cells preparation (lane 1), SDS extract (lane 2), WP from phenol extraction of the SDS extract (lane 3), PP from phenol extraction of the SDS extract (lane 4). Size markers (K values, lane M). Proteins and LPS stained with CBB and silver, respectively.

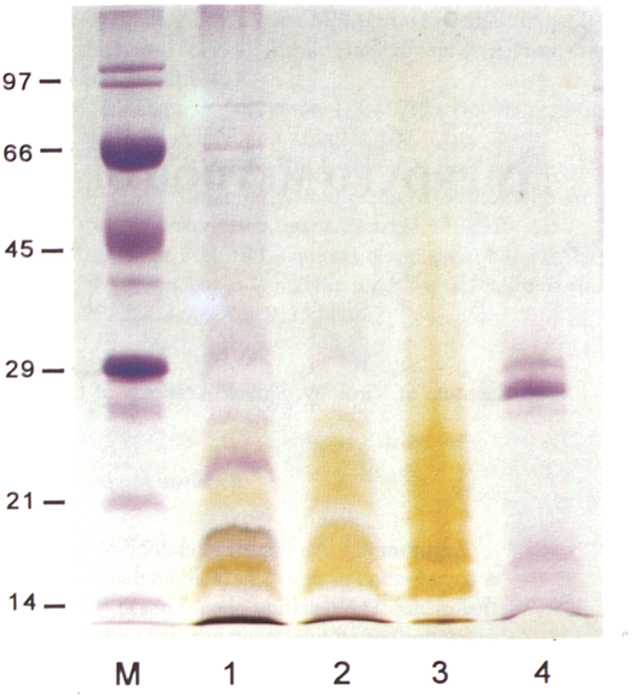

Fig. 2
SDS-PAGE of proteins and LPS extracted from *C. burnetii* with DOC and phenol
Whole cells preparation (lane 1), DOC extract (lane 2), WP from phenol extraction of the DOC extract (lane 3), PP from phenol extraction of the DOC extract (lane 4). For the rest of legend see Fig. 1.

pH 8.0 C for 2 hrs at 37°C (a modifited method of Caldwell *et al.*, 1981). The suspension was then centrifuged at 18,000 x g for 1 hr, the pellet was once more extracted for 1 hr, and the pooled supernatants were dialyzed against tap and distilled water and lyophilized. The lyophilized SDS or DOC extracts were dissolved in distilled water and treated with and equal volume of 90% phenol under stirring for 10 mins at 68°C (Westphal and Jann, 1965). After a low-speed centrifugation the WP was saved, while the PP was twice more extracted with water under the same conditions. The pooled WP and PP were each separately dialyzed against distilled water. All the preparations (WP SDS, PP SDS, WP DOC, PP DOC) were analyzed by SDS-PAGE.

SDS-PAGE was carried out according to Laemmli (1970) with 12% and 5% acrylamide in separating and stacking gels, respectively. The gels were silver-stained for LPS (Tsai and Frasch, 1982), and then by Coomassie Brilliant Blue R 250 (CBB) for proteins. 100 µg aliqouts of lyophilized materials were applied on gels throughout.

Immunoblot analysis was performed according to Towbin *et al.* (1979). Blots were reacted with corresponding mouse or rabbit sera (1:400) in 10% non-fat dry milk. The reaction was visualized with horseradish peroxidase-conjugated secondary antibodies (1:500) and 4-chloro-1-naphtol. Mice were immunized i.p. with one dose (50 µg) of purified *C. burnetii* proteins. The blood was collected and pooled 1 month after immunization. Rabbits were immunized i.v. with 50, 100, 200, and 500 µg doses of the same preparations on days 0, 4, 7, 11, and 51. The blood was collected one week after the last dose.

Results and Discussion

The detergent extracts of phase I *C. burnetii* corpuscles contained both proteins and LPS (Figs. 1 and 2) similarly to those obtained by TCA (Lukáčová *et al.*, 1989) or Empigen BB (Lukáčová *et al.*, 1994) treatment. Thus all these procedures yielded the *C. burnetii* the outer membrane complex consisting of proteins and LPS detectable in SDS-PAGE, though the composition of the protein fraction differed in dependence on the procedure used. The TCA extract contained mainly low molecular mass proteins below 20 K, while the Empigen BB extract contained mainly 29 K protein and some other proteins of various molecular mass. SDS and DOC extracted a broader

spectrum of proteins than the two other methods, but the most expressed bands were in each case in the range of about 30 K. In all extracts LPS exhibited the same SDS-PAGE pattern. The hot phenol procedure applied to the detergent extracts resulted in separation of LPS into the WP and proteins into the PP (lanes 3 and 4, respectively, in Figs. 1 and 2). Lane 3 in Fig. 1 was overloaded with 100 μg of lyophilized LPS to visualize better the 34 K and 29 K proteins of the WP. There was no substantial difference between the SDS and DOC extracts as regards their yield and composition. The SDS and DOC extracts represented 24.2% and 22.3% of the *C. burnetii* whole cells mass, respectively. SDS WP, SDS PP, DOC WP and DOC PP represented 3.5%, 3.75%, 3.25%, and 5.85% of *C. burnetii* whole cells mass, respectively.

The SDS-PAGE showed that it was the DOC extract only that yielded a LPS-free protein fraction in the PP. Proteins in the PP obtained from the SDS extract contained also a small amount of LPS (Fig. 1, lane 4). Either extract led to PP containing three proteins of 29 K, 26 K, and 24 K, of which the 26 K was predominant. The WP obtained from the SDS extract contained besides LPS also two proteins of 34 K and 29 K. The *C. burnetii* 29 K protein was apparently tightly bound to LPS, forming a LPS-protein complex. This fact may explain why even a purified protein evoked antibodies against LPS when injected into laboratory animals (Williams *et al.*, 1990).

Fig. 3 shows the immunoblot analysis of immune mouse (lane 1) and rabbit (lane 2) sera with DOC PP as antigen. Whereas the mouse serum recognized the 14 K, 21 K and 66 K proteins, the rabbit serum did a broad spectrum of proteins with predominance of the 21 K protein.

A rabbit serum against the DOC PP did not react in immunoblot analysis with phase I *C. burnetii* LPS (data not shown).

Our results show that it is possible to isolate from phase I *C. burnetii* cells a protein fraction by use of detergent – phenol extraction procedures. This fraction is capable to evoke an antibody response in laboratory animals. In the outer membrane of *C. burnetii* the 29 K protein apparently forms a complex with LPS.

It seems that the LPS-protein complex plays an important role in defense reactions of *C. burnetii* cells and is therefore the strongest immunogenic structure present in its outer membrane.

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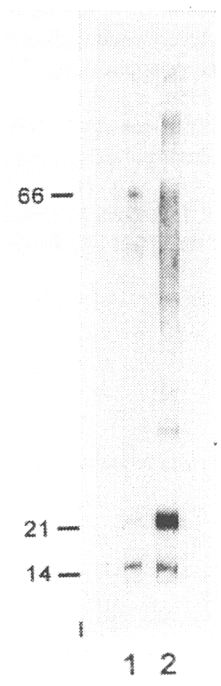

Fig. 3
Immunoblot analysis of PP from phenol extraction of DOC extract of *C. burnetii*
Mouse (lane 1) and rabbit (lane 2) immune sera. K values of size markers are indicated on the left side.

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