IMMUNOLOGICAL CONSEQUENCES OF *COXIELLA BURNETII* PHASE VARIATION

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Summary. – The influence of the number of passages in chick embryo yolk sac (EPs) on the properties of the lipopolysaccharide (LPS) and other antigens of *Coxiella burnetii* Priscilla strain in polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE), immunoblot analysis, enzyme-linked immunosorbent assay (ELISA) and complement-fixation reaction (CFR) test has been studied. Three phases in the phase variation of *Coxiella burnetii* could be distinguished by these methods: phase I lasting up to the 20th passage (EP 20), intermediate phase corresponding to EP 20-EP 70, and phase II beginning at EP 80. The changes in LPS were more marked than those in proteins which conserved their immunoblot profile up to EP 80. The phase II was clearly demonstrated by all the methods used.

Key words: Coxiella burnetii; phase variation; lipopolysaccharide; antigens

Introduction

C. burnetii, the ethiological agent of Q fever, is an obligatory intracellular parasite which undergoes a unique phase variation during passaging in chick embryo yolk sac. There occurs a LPS transition from phase I with complete saccharidic side-chain through an intermediate phase to phase II with truncated LPS without saccharidic side-chain (Schramek and Mayer, 1982). This transition is similar to the S-R variation of *Enterobacteriaceae*. The biosynthesis of LPS core and the O-chain in many Gram-negative bacteria is controlled by genes organised in the *rfa*-gene cluster. The synthesis of the side-chain of LPS in *Escherichia coli* is regulated by a protein encoded by *rol* gene (Schnaitman and Klena, 1993). The whole system is highly conserved. However, a sequence homologous to the *rol* gene of *E. coli* was not found in the *C. burnetii* DNA (Valková *et al.*, 1996). It seems that the regulation of the LPS side-chain synthesis in *C. burnetii* differs from that in enterobacteria. The transition from phase I to II in *C. burnetii* strains Nine Mile and Henzerling proceeded through an intermediate phase II: the phase I represented EPs 1-10, the intermediate phase II EPs 15-45, and the phase II EP 75 and higher (Višacká *et al.*, 1984). Immunological studies on EPs 3, 10, 20, and 30 of *C. burnetii* Priscilla strain showed differences between EPs 3 and 10 (phase I), and EPs 20 and 30 (intermediate phase II) (Lukáčová *et al.*, 1993). Priscilla is one of the most peculiar strains of *C. burnetii* which has been considered the representative of endocarditis-associated strains with special type of plasmid (Samuel *et al.*, 1985), different LPS profiles in SDS-PAGE (Hackstadt, 1986) and low virulence for laboratory animals (Moos and Hackstadt, 1987). However, the PCR study of different acute and chronic isolates showed that the nature of the host, rather than that of the pathogen, was crucial for Q fever endocarditis (Thiele and Willems, 1994). From this point of view it was interesting to study the changes of LPS of Priscilla strain during chick embryo yolk sac passaging up to the complete transition to the phase II. Phase I LPS of *C. burnetii* represents an important virulence factor similarly to LPSs of other Gram-negative bacteria. With higher passages, the virulence of *C. burnetii* drops; the less

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Abbreviations: CFR = complement-fixation reaction; ELISA = enzyme-linked immunosorbent assay; EP = chick embryo yolk sac passage; LPS = lipopolysaccharide; SDS = sodium dodecyl sulphate; SDS-PAGE = polyacrylamide gel electrophoresis in the presence SDS

Table 1. ELISA and CFR test of various passages of *C. burnetii* Priscilla strain and their respective antisera

C. burnetii Priscilla strain passage	Titres of antisera					
	Anti-EP 3		Anti-EP 50		Anti-EP 80	
	CFR	ELISA	CFR	ELISA	CFR	ELISA
EP 3	128	>409,600	128	12,800	0	6,400
EP 50	32	409,600	128	25,600	0	6,400
EP 80	32	800	8	12,800	16	25,600

virulent rickettsia does not resist the host immune system (Kazár *et al.*, 1975). The phase variation of *C. burnetii* is a phenomenon including changes in its virulence, serological, biological, physical, chemical and antigenic properties (Kazár *et al.*, 1974). It seems that *C. burnetii* expends energy for the synthesis of the whole-chain LPS only when this chain is necessary for its defence against the host immune system.

Materials and Methods

C. burnetii strain Priscilla, kindly supplied by M. Peacock the Rocky Mountain Laboratories, Hamilton, MT, USA, was passaged in chick embryo yolk sac and purified by differential centrifugation and ether treatment (Ormsbee, 1962). The EPs 80 and 90 were purified also by a trypsin treatment (Úrvölgyi, 1976). The EPs 3, 40, 50, 60, 70, 80, and 90 were compared. Whole-cell antigen preparations consisted of rickettsial purificates solubilised according to Laemmli (1970).

LPS isolation. Purified rickettsial EPs (2 mg/ml) were solubilised with a lysis buffer (2% SDS, 4% 2-mercaptoethanol, 10% glycerol, 1 mol/l Tris.HCl pH 6.8, and bromphenol blue) at 100°C for 5 mins, and incubated with proteinase K at 60°C for 1 hr. LPS was precipitated with 2 volumes of 0.375 mol/l $MgCl_2$ in 95% ethanol at -20°C overnight (Jürgens and Fehrenbach, 1997).

ELISA was performed according to Brade *et al.* (1994). Whole-cell antigens were used. Twofold dilutions of immune sera were used in the assay and the reciprocal of the last dilution yielding A_{405} >0.2 was taken for the serum titre. When duplicates in both tests differed by more than one dilution, a third determination was performed.

CFR test was done according to Brezina (1958).

SDS-PAGE of LPS was carried out according to Laemmli (1970) on 15% acrylamide gels. Ten μg of LPS per lane was used. The gels were silver-stained (Hitchcock, 1983).

Immunoblot analysis of whole-cell antigen and LPS preparations was performed according to Towbin *et al.* (1979). Blots on nicrocellulose membranes were developed with corresponding hyperimmune rabbit sera diluted to 1:200 and 1:400 in 10% non-fat dry milk. The reaction was visualised with horseradish peroxidase-conjugated swine anti-rabbit IgG (1:500) and 4-chloro-1-naphtol as substrate.

Hyperimmune rabbit sera were obtained by immunisation of rabbits intravenously with 50, 100, 200, and 500 μg doses of EPs 3, 50, and 80 (corpuscular antigens) on days 0, 4, 7, 11, and 51. The blood was collected one week after the last dose.

Results

The results of the reaction of EPs 3, 50 and 80 of *C. burnetii* Priscilla strain with their respective antisera in ELISA and CFR test are shown in Table 1.

In CFR, EP 3 antigen reacted similarly with both the EP 3 and EP 50 antisera but did not with EP 80 antiserum. Using EP 50 antigen, EP 3 antiserum exhibited a lower titre than EP 50 antiserum, while EP 80 antiserum did not react at all. Using EP 80 antigen, EP 3 and EP 80 antisera gave similar titres, indicating that the phase II antigen shared some common antigenic epitopes with the phase I antigen.

In ELISA, titres of EP 3, EP 50 and EP 80 antisera against EP 3 antigen were decreasing (>409,600, 12,800 and 6,400, respectively). The same antisera, when tested against EP 50 antigen (intermediate phase antigen) gave decreasing titres of 409,600, 25,400 and 6,400, respectively; however, when tested against EP 80 antigen, they showed rising titres of 800, 12,800 and 25,600, respectively. These results except the titre of 800 (regarded as negative) indicated common epitopes on all the antigens tested.

Therefore, we propose the following designation of phases in the serological phase variation of *C. burnetii* Priscilla strain: the phase I (EP 3 – EP 10), the intermediate phase (EP 20 – EP 70) instead of the intermediate phase II of Višacká *et al.* (1984), and the phase II (EP 80 and higher passages).

SDS-PAGE profiles of LPSs of *C. burnetii* Priscilla strain EPs 50, 80 and 90 (Fig. 1) indicated a great change between EP 50 and EP 80, supporting our abovementioned proposal. The profiles of EP 80 and EP 90 were similar and demonstrated that the transition from the phase I to II was completed. The purity of the LPS preparations used, especially the absence of proteins was proved by negative results of the staining of the gels with Coomasie Brilliant Blue for proteins (results not shown).

Whole-cell antigen (Figs. 2-4) and LPS preparations (Figs. 5-7) from EP 3 – EP 90 were subjected to the immunoblot analysis using the EP 3, EP 50 and EP 80 antisera for detection. The EP 3 and EP 50 antisera reacted with all the EP antigens except EP 80 and EP 90 (Figs. 2 and 3). The most marked proteins were of about 65 K and 54 K. The profiles of the antigens EP 40 – EP 70 were very similar. EP 80 antiserum reacted similarly with all the

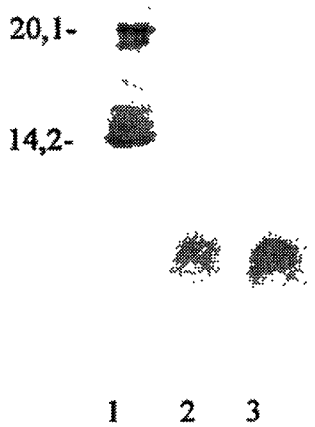

Fig. 1
SDS-PAGE of LPS praparations from various passages of *C. burnetii* Priscilla strain
EP 50 (lane 1), EP 80 (lane 2), EP 90 (lane 3).

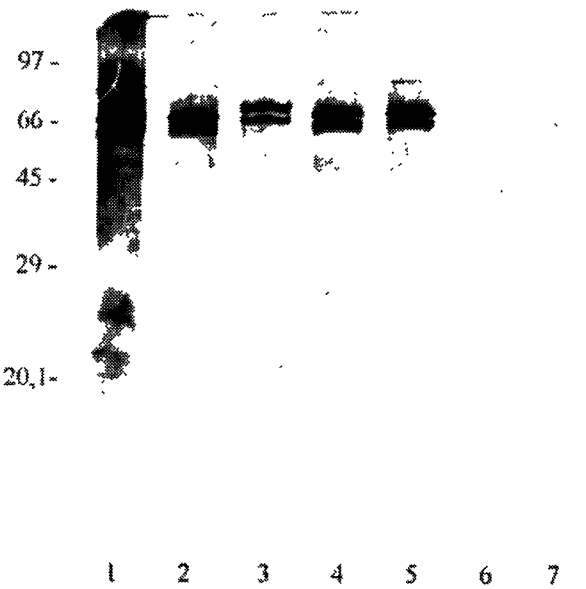

Fig. 2
Immunoblot analysis of *C. burnetii* whole-cell antigens from various passages
EP 3 antiserum used for detection. Antigens EP 3 (lane 1), EP 40 (lane 2), EP 50 (lane 3), EP 60 (lane 4), EP 70 (lane 5), EP 80 (lane 6), and EP 90 (lane 7).

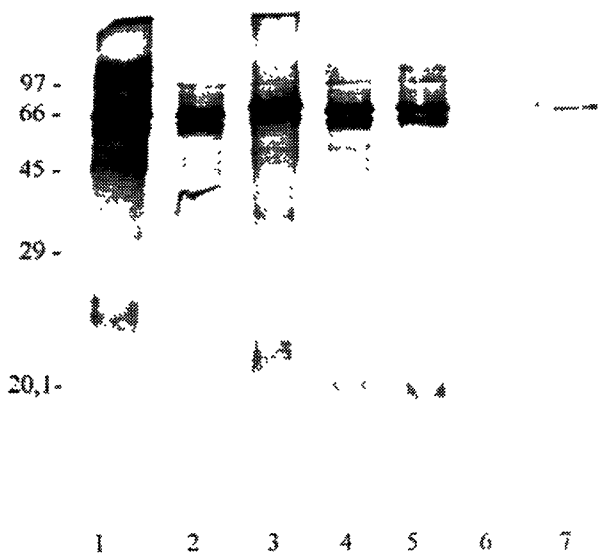

Fig. 3
Immunoblot analysis of *C. burnetii* whole-cell antigens from various passages
EP 50 antiserum used for detection. For the lanes see Fig. 2.

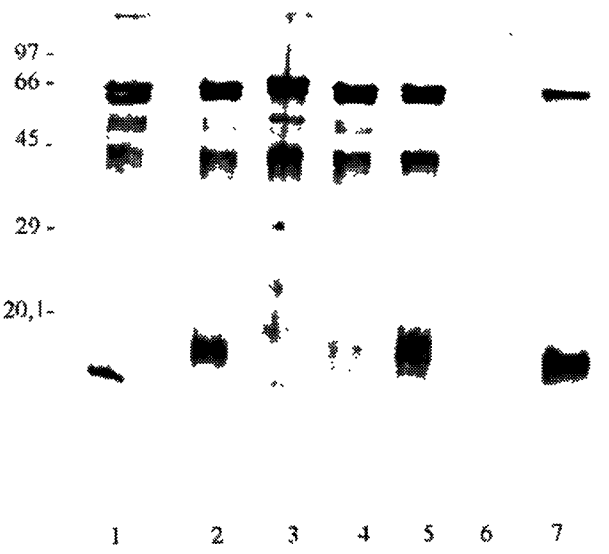

Fig. 4
Immunoblot analysis of *C. burnetii* whole-cell antigens from various passages
EP 80 antiserum used for detection. For the lanes see Fig. 2.

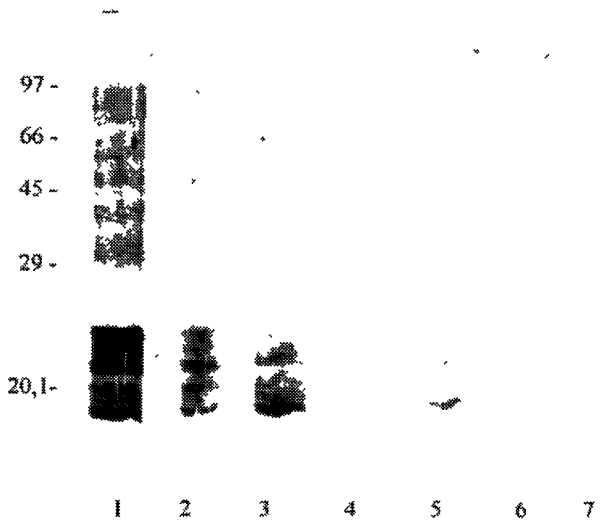

Fig. 5
Immunoblot analysis of *C. burnetii* LPSs from various passages
EP 3 antiserum used for detection. For the lanes see Fig. 2.

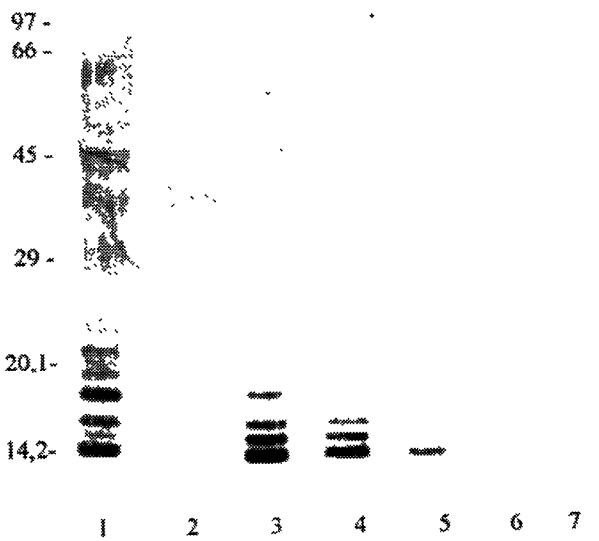

Fig. 6
Immunoblot analysis of *C. burnetii* LPSs from various passages
EP 50 antiserum used for detection. For the lanes see Fig. 2.

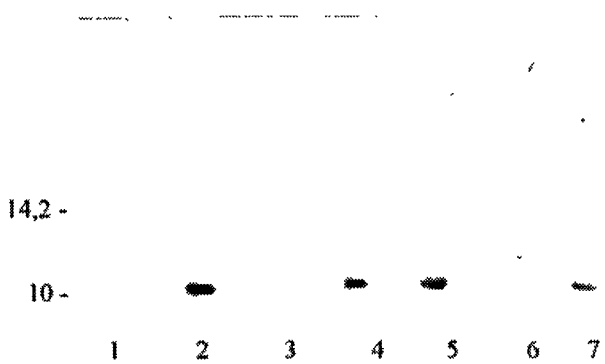

Fig. 7
Immunoblot analysis of *C. burnetii* LPSs from various passages
EP 80 antiserum used for detection. Fot the lanes see Fig. 2.

antigens from all passages except EP 80 and EP 90 (Fig. 4). The intensity and number of LPS bands decreased with higher passage (Figs. 5-7), so that finally only a 10 K band representing the phase II LPS could be seen.

Discussion

C. burnetii, an obligatory intracellular parasite, differs from other Gram-negative bacteria in its developmental cycle in the phagolysosome of eukaryotic host cells. After multiplication, *C burnetii* particles are present in the host cell only in low number and display pleomorphism. Therefore, it is very difficult to prepare a sufficient amount of this parasite and particularly its LPS for experimental work. To isolate LPSs of different EPs of *C. burnetii* for SDS-PAGE and immunoblot analysis, we used the method of Jürgens and Fehrenbach (1997) elaborated for *Legionella* species. We have studied the changes in the isolated LPSs during passaging by various serological methods. The transition of the LPS from the intermediate phase to phase II showed a marked change in the number of repeating O-chain units from EP 50 to EP 80 in SDS-PAGE. We were able to determine various phases in this process. In CFR test, the antiserum against the phase II antigen did not react with the phase I and intermediate phase antigens, but the antiserum against the phase I antigen reacted with the phase II antigen, indicating some common protein epitopes exposed after the O-chain removal. In ELISA, the highest titres were obtained with the EP 3 antiserum (against the phase I antigen) and homologous antigen, but the titre of this antiserum with the intermediate EP 50 antigen was also very high. Serological results showed a relationship of the antigens of the phase I and intermediate phase, and confirmed some common epitopes in the antigens in these two phases.

In the immunoblot analysis, the phase I (EP 3) and intermediate phase (EPs 20 – 70) antigens reacted similarly with EP 3 and EP 50 antisera. The profiles of the antigens of EPs 3, 40, 50, 60, and 70 with these two antisera are similar, indicating an immunological relationship of proteins in the phase I and intermediate phase. On the other hand, EP 3 antiserum (phase I) and EP 50 antiserum (intermediate phase) did not react with phase II proteins (EP 80 and EP 90). However, this result differed from those obtained

in ELISA. The most immunogenic proteins of these EPs were two, probably heat shock proteins of 68 K and 54 K. EP 80 antiserum (phase II) reacted with proteins of all rickettsial EPs. However, the profiles of EP 80 and EP 90 differed probably due to different preparation (trypsin treatment). In LPS immunoblots, antisera against the phase I and intermediate phase antigens distinguished the ladder-like LPS structure of EPs 3 – 70 with decreasing number of saccharide units but did not that of EP 80 and EP 90. EP 80 antiserum distinguished one band of phase II LPS only in intermediate phase LPS but not in phase I LPS.

The transition of *C. burnetii* Priscilla strain from phase I through intermediate phase to phase II proceeded in the same way as that of other *C. burnetii* strains, Henzerling and Nine Mile, as estimated by other serological methods (Višacká *et al.*, 1984). Phase I passed to intermediate phase by EP 20; from EP 20 to EP 70, there was intermediate phase, and phase II began by EP 80. We did not try to revert intermediate phase to phase I by passaging in mice; this passaging is known to revert the lower virulence of this phase to full virulence of phase I (Brezina, 1958). However, we can assume that the Priscilla strain like other *C. burnetii* strains cannot be reverted in mice from phase II to I (Brezina, 1977). The impossibility of this phase reversal in mice supports our proposal to divide the *C. burnetii* phases in phase I, intermediate phase (with relation to phase I) and phase II which exhibits all properties of a laboratory mutant without possibility of its own life in immunologically competent hosts. The relatively lower virulence of Priscilla strain (Moos and Hackstadt, 1987) did not influence the phase variation of this strain. To date, all *C. burnetii* strains studied showed the same mechanism of phase variation from phase I with full O-chain LPS through intermediate phase with a reduced LPS ladder to phase II with truncated LPS (without O-chain saccharides).

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