INFECTION AND IMMUNITY, May 1985, p. 359–365
0019-9567/85/050359-07$02.00/0

Vol. 48, No. 2

Lipopolysaccharide Variation in *Coxiella burnetii*: Intrastrain Heterogeneity in Structure and Antigenicity

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Received 26 November 1984/Accepted 15 January 1985

We isolated lipopolysaccharides (LPSs) from phase variants of *Coxiella burnetii* Nine Mile and compared the isolated LPS and *C. burnetii* cells by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting. The LPSs were found to be the predominant component which varied structurally and antigenically between virulent phase I and avirulent phase II. A comparison of techniques historically used to extract the phase I antigenic component revealed that the aqueous phase of phenol-water, trichloroacetic acid, and dimethyl sulfoxide extractions of phase I *C. burnetii* cells all contained phase I LPS, although the efficiency and specificity of extraction varied. Our studies provide additional evidence that phase variation in *C. burnetii* is analogous to the smooth-to-rough LPS variation of gram-negative enteric bacteria, with phase I LPS being equivalent to smooth LPS and phase II being equivalent to rough LPS. In addition, we identified a variant with a third LPS chemotype with appears to have a structural complexity intermediate to phase I and II LPSs. All three *C. burnetii* LPSs contain a 2-keto-3-deoxyoctulosonic acid-like substance, heptose, and gel *Limulus* amoebocyte lysates in subnanogram amounts. The *C. burnetii* LPSs were nontoxic to chicken embryos at doses of over 80 μg per embryo, in contrast to *Salmonella typhimurium* smooth- and rough-type LPSs, which were toxic in nanogram amounts.

Coxiella burnetii, the etiologic agent of Q fever, is an obligately intracellular bacterium that multiplies within the phagolysosome of eucaryotic cells (8, 18, 19). *C. burnetii* is unique among the rickettsiae in that it undergoes what has been termed phase variation (3, 12, 39). Virulent phase I is isolated from natural and laboratory infections. Conversion to avirulent phase II occurs during serial passage in nonimmunologically competent hosts such as embryonated eggs or tissue culture (3, 4, 12).

Designation of phase is serological and based on the ability of the organism to bind early (<20 days postvaccination) antisera. Organisms that react with early antisera are considered to be phase II. Those that react only with late sera are considered to be phase I (12, 39). Both anti-phase I and anti-phase II antibodies are produced in response to phase I infection or vaccination, but only phase II reactive antibodies are produced in response to phase II infection or vaccination. The phase I antigen may be removed with trichloroacetic acid (TCA) (7, 14) or periodate, or by mild acid hydrolysis (35), leaving a particulate residue with only phase II reactivity. The phase I antigenic component has thus been thought to mask the phase II antigenic determinant(s) (3, 7, 35).

Differences in surface properties between the virulent and avirulent phases (3, 13, 22) and the carbohydrate nature of the phase I antigen (7, 13) have long led to analogies between this phase variation and the smooth-to-rough transition of various bacteria (13, 37). With the demonstration of qualitative and quantitative differences in the sugar composition of lipopolysaccharides (LPSs) isolated from the two phases (37), it appears that phase variation in *C. burnetii* parallels the smooth-to-rough variation of gram-negative enteric bacteria.

We began isolating *C. burnetii* LPSs in attempts to define the virulence components of this organism. Our results clearly support analogies (37) between phase variation of *C. burnetii* and smooth-to-rough LPS transitions. In addition, we identified a third LPS chemotype which appears to have a structural complexity intermediate to phase I and II LPSs.

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MATERIALS AND METHODS

Organisms. *C. burnetii* Nine Mile in phase I (9 mi/I) was passed 306 times in guinea pigs, plaque purified (31) from primary chicken embryo cell cultures (clone 7), and passed three times in chicken embryos (306 GP, 1 TC, 3 EP). Phase II *C. burnetii* Nine Mile (9 mi/II) was passed 94 times in chicken embryos, plaque purified from primary chicken embryo cell cultures (clone 4), and passed three times in chicken embryos (94 EP, 1 TC, 3 EP). The third *C. burnetii* Nine Mile (9 mi/Cr) was passed 306 times in guinea pigs and 4 times in chicken embryos, and then was used to infect a guinea pig. After 343 days, the guinea pig was sacrificed, and 9 mi/Cr was isolated from placental tissue by three passes in chicken embryos (306 GP, 4 EP, 1 GP 343 days, 3 EP).

C. burnetii was propagated in specific pathogen-free type IV, antibiotic-free fertile hen's eggs (H & N Hatchery, Redmond, Wash.) and purified by a procedure that includes two cycles of Renografin (E. R. Squibb & Sons, Princeton, N.J.) density gradient centrifugation (48).

Salmonella typhimurium SCSG225 (smooth LPS chemotype) and *S. typhimurium* SAI377 (Re LPS chemotype) were obtained from Ken Sanderson, Salmonella Genetic Stock Center, Calgary, Alberta, Canada.

Antisera. Hyperimmune rabbit antisera were prepared against the cloned isolates of 9 mi/I and 9 mi/II by an initial vaccination with 300 μg (dry weight) of Formalin-killed organisms. The animals were given booster immunizations three times with the respective antigen, and sera were prepared 12 days after the final immunization.

SDS-PAGE and immunoblotting. Procedures for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), immunoblotting, and surface iodination were as

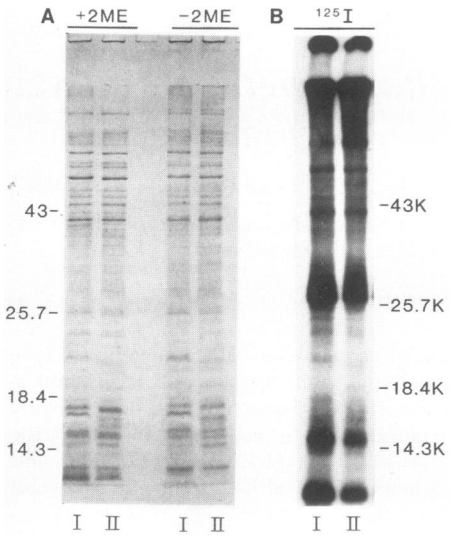

FIG. 1. (A) Comparison of *C. burnetii* Nine Mile phase I and II by SDS-PAGE and Coomassie brilliant blue staining, and (B) an autoradiogram of lactoperoxidase-catalyzed surface radioiodinated *C. burnetii* phases I and II. The acrylamide concentration was 12.5% for all panels of this figure only. *C. burnetii* whole-cell lysates were solubilized for electrophoresis in the presence or absence of 2-ME as described (17). Molecular weight markers ($\times 10^3$) appear on the left and right of the gels.

previously described (17). Silver staining was as described by Tsai and Frasch (41) as modified by Hitchcock and Brown (20).

LPS isolation. LPS was extracted from purified *C. burnetii* Nine Mile by a modification of the hot phenol-water method of Westphal and Jann (47). The procedure was modified because the extracted LPS, in particular phase I LPS, was very soluble in water (>10 mg/ml of H_2O) and only small amounts were precipitated by centrifugation at 80,000 and 105,000 $\times$ *g*. Because of the poor efficiency of LPS purification by centrifugation, the aqueous phase of the extract was dialyzed extensively, lyophilized, suspended in 10 mM Tris–2 mM $MgCl_2$–150 mM NaCl[pH 8.0] plus DNase I and RNase A (Sigma Chemical Co., St. Louis, Mo.), each at 200 μg/ml, and incubated for 16 h at 37°C with shaking. The digests were reextracted with phenol at 68°C, and the aqueous phase was dialyzed and lyophilized. This nuclease treatment failed to digest all contaminating RNA. RNA contamination of the LPS preparations treated in this manner varied from about 5 to 30% by weight. Because even grossly contaminated LPS preparations (~60% RNA) behaved on SDS-PAGE and immunoblotting procedures identically to the purest preparations, the LPSs prepared in this manner were judged suitable for the structural and immunological analysis presented here. *S. typhimurium* LPSs were extracted by the same procedures. In some experiments, the phenol phase was saved, dialyzed extensively, and lyophilized.

Other procedures for extraction of the phase I antigen included the use of dimethyl sulfoxide (DMSO) as described by Ormsbee et al. (29). Briefly, 100 mg (dry weight) of purified 9 mi/I was suspended in 100 ml of DMSO, shaken at 50°C for 24 h, and centrifuged at 5,000 $\times$ *g* for 60 min. The supernatant was then dialyzed extensively and lyophilized. The phase I antigen was also extracted with TCA, similar to the method described by Brezina and Urvolgyi (7). A sample (100 mg [dry wt]) of 9 mi/I was suspended in 100 ml of 10% (wt/vol) TCA and held at 4°C for 4 h with occasional mixing. The suspension was pelleted at 16,000 $\times$ *g* for 30 min, and the supernatant was then dialyzed and lyophilized.

Chemical analysis. Protein concentrations were determined by the method of Bradford (5) with Bio-Rad (Bio-Rad Laboratories, Richmond, Calif.) reagents and bovine serum albumin as a standard. RNA was quantified by the orcinol reaction with yeast RNA (Sigma) as a standard. Values obtained for nucleic acid contamination of LPS preparations by optical density at 260 nm, also with yeast RNA as a standard, were similar to those obtained by the orcinol reaction. The 2-keto-3-deoxyoctulosonic acid (KDO) content of the LPS was assayed by the method of Weisbach and Hurwitz (44) as modified by Osborn (32). Heptoses were quantified by the cysteine-sulfuric acid method of Dische (10) as modified (32) with D-mannoheptulose as a standard. Total carbohydrate was determined by the phenol-sulfuric acid method of Dubois et al. (11) with glucose as a standard.

Biological assays. Toxicity of the isolated LPSs was assayed by chicken embryo lethality (27) in specific pathogen-free eggs (utility grade, SPAFAS, Inc., Norwich, Conn.). The 50% lethal dose for chicken embryos was estimated by the method of Reed and Meunch (33). The ability of the LPSs to gel *Limulus* amoebocyte lysates (LALs) was assayed by Pyrogent (Mallinckrodt, Inc., St. Louis, Mo.) LAL, according to instructions provided.

RESULTS

Polypeptide profiles of phases I and II. The protein compositions of phases I and II *C. burnetii* Nine Mile were first compared by SDS-PAGE, followed by Coomassie brilliant blue staining (Fig. 1). Samples were solubilized in either the presence or the absence of 2-mercaptoethanol (2-ME) before SDS-PAGE. Although the *C. burnetii* polypeptides resolved somewhat more sharply in the presence of 2-ME, dramatic differences in the solubility or migration of predominant proteins in the presence or absence of 2-ME were not evident. In general, the polypeptide profiles of phase I and phase II *C. burnetii* were strikingly similar. Some differences were seen in the apparent molecular weight of proteins in the range of 14,000 to 25,700.

A preliminary analysis of surface-exposed proteins was made by comparison of the proteins labeled by lactoperoxidase-catalyzed radioiodination of *C. burnetii* in phase I or phase II (Fig. 1B). As in the Coomassie brilliant blue-stained gel profile above, some differences in the migration of ^{125}I-surface-labeled proteins on SDS-PAGE were apparent in the molecular weight range of 14,000 to 25,700. We have not explored these apparent phase-related differences in migration of polypeptides in greater detail because alternative explanations for the apparent differences in one-dimensional SDS-PAGE profiles are possible. This will be discussed.

LPS of phase I and phase II *C. burnetii*. We modified the hot phenol-water procedure of Westphal and Jann (47) to extract LPSs from the cloned isolates of 9 mi/I and 9 mi/II. The isolated LPSs were analyzed by SDS-PAGE with silver staining to show LPS species. The isolated LPSs were compared to whole-cell lysates and proteinase K digests of whole-cell lysates, a method previously used to compare LPS chemotypes from members of the family *Enterobacteriaceae* (20). The antigenicity of the *C. burnetii* LPSs and of protein antigens was examined by immunoblotting with hyperimmune rabbit antisera prepared for cloned 9 mi/I and 9 mi/II.

Several points may be made from the data shown in Fig. 2.

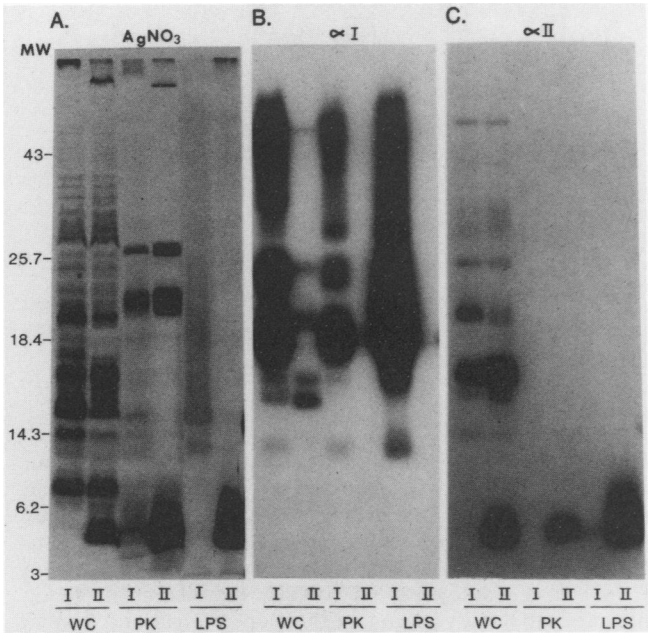

FIG. 2. SDS-polyacrylamide gel profile and accompanying immunoblots of *C. burnetii* Nine Mile phases I and II, clone 7 and clone 4, respectively. Panels A, B, and C were run in parallel on 15% polyacrylamide gels and (A) stained by the silver nitrate procedure, (B) immunoblotted against rabbit hyperimmune antisera prepared against 9 mi/I, or (C) immunoblotted with hyperimmune rabbit antisera to 9 mi/II. In each of the panels are whole-cell lysates (WC) of 9 mi/I and 9 mi/II, proteinase K digests of whole-cell lysates (PK) prepared as previously described (20), and isolated LPS from 9 mi/I and 9 mi/II. The LPSs, particularly those from 9 mi/I and 9 mi/II, stained bright yellow by the silver staining procedure and thus are somewhat poorly reproduced in the black-and-white photographs of the proteinase K digests here and in Fig. 3 and 4. Note the banding pattern of phase I LPS in the immunoblot near the regions between the 25,700- and extending above the 43,000-molecular-weight markers. These antigenic species do not appear to stain with the silver staining procedure. Note also that only three or four of the five prominent silver-staining bands in the phase I LPS reacted in the immunoblots. The position of molecular-weight protein markers (MW [$\times 10^3$]) are indicated on the left. Although the relative migration of LPS species is believed to reflect molecular weight (41), the differential binding of SDS to LPS versus protein (28) does not allow direct comparison of LPS migration to the migration of protein molecular-weight standards. Markers are thus included for reference only.

(i) The differences in the polypeptide profiles of phase I and II *C. burnetii* seen in the Coomassie brilliant blue-stained PAGE profile in the 14,000- to 25,700-molecular-weight range were accentuated by the silver staining procedure. (ii) There are several antigens shared between the two phases, although they vary somewhat between phases in intensity of reaction after immunoblot. (iii) These shared antigens were not detected in the proteinase K-digested lysates or the isolated LPSs and thus probably represent protein antigens. (iv) The predominant components antigenically unique by this technique were the LPSs. The LPS of each phase is structurally unique by SDS-PAGE and was recognized in immunoblots only by the homologous antisera.

The nature of the darkly staining bands in the proteinase K digests just above and below the 25,700-molecular-weight markers (Fig. 2A) is unknown but of rickettsial origin because the bands are not seen in control lanes containing

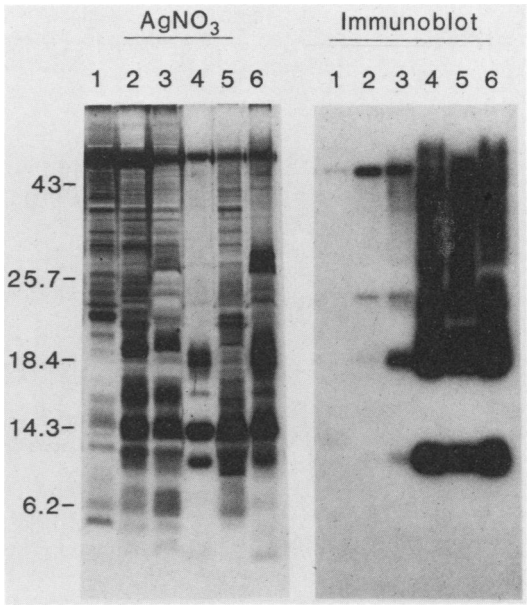

FIG. 3. Comparison of *C. burnetii* phase I antigen extraction procedures by SDS-PAGE with silver-staining and a parallel immunoblot reacted with pooled rabbit anti-*C. burnetii* 9 mi/I and 9 mi/Cr antisera. Lanes: 1, phenol phase of the hot phenol-water extraction; 2, insoluble pellet after DMSO extraction; 3, insoluble pellet after TCA extraction; 4, aqueous phase of the hot phenol-water extraction; 5, aqueous phase after DMSO extraction; and 6, aqueous phase after TCA extraction. Molecular weight markers ($\times 10^3$) appears on the left.

self-digested proteinase K. These components appeared similar in migration on SDS-PAGE between phases, although there seemed to be somewhat more in the phase II digests. The appearance of these components on silver-stained SDS gels of proteinase K-digested whole-cell lysates of *C. burnetii* was in contrast to proteinase K digestion of *Salmonella*, from which only LPS with migration similar to that of purified LPS was seen on silver-stained gels (20). Note also the components near the top of the gel in the proteinase K digests that varied between phases I and II.

Comparison of phase I antigen (LPS) extraction procedures. The phase I antigen of *C. burnetii* was extracted by a variety of procedures, including hot phenol-water (6), TCA (7), and DMSO (29). We compared the relative efficiency of phase I antigen extraction by each of these procedures. The aqueous extracts from each of them and from the insoluble precipitates (or phenol phase) were examined by SDS-PAGE and

TABLE 1. Comparison of extraction procedures for *C. burnetii* phase I antigen (LPS)

Extraction procedure	Yield (mg)[a]	Protein (%)[b]	RNA (%)[b]
H_2O phase	16.6	<1.0	57.7
Phenol phase	15.8	33.7	10.9
TCA supernatant	7.1	5.6	11.3
TCA pellet	94.0	32.1	6.9
DMSO supernatant	32.3	23.2	10.7
DMSO pellet	71.0	18.6	6.3

[a] Yield is expressed as mg (dry weight) recovered in each phase from 100 mg (dry weight) of *C. burnetii* Nine Mile phase I cells.

[b] Percent dry weight. No additional extractions or treatments were performed on the materials extracted by each of these procedures.

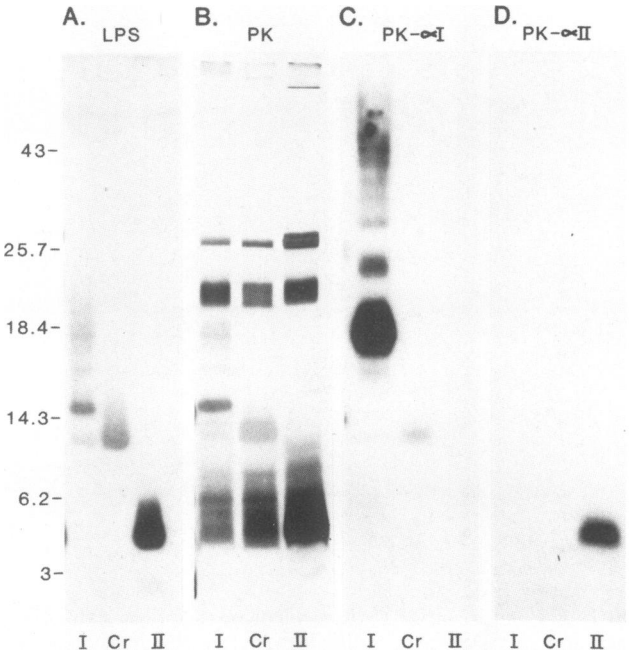

FIG. 4. Silver-stained PAGE profile of isolated LPS (A) and proteinase K digests (B) of *C. burnetii* 9 mi/I, 9 mi/Cr, and 9 mi/II and accompanying immunoblots of proteinase K (PK) digests of each of the three LPS chemotypes developed with rabbit hyperimmune sera against phase I (C) or phase II (D) *C. burnetii*. The acrylamide concentration was 15% in each of the gels. The LPSs, particularly those from 9 mi/I and 9 mi/Cr, stained bright yellow by the silver staining procedure and thus are somewhat poorly reproduced in the black-and-white photographs of the proteinase K digests here and in Fig. 2. The small peptides remaining after proteinase K digestion migrated near the dye front, obscuring the phase II LPS. Note the banding pattern of phase I LPS in the immunoblot near the regions between the 25,700- and 43,000-molecular-weight markers. These antigenic species do not appear to stain with this silver staining procedure. The nature of the dark staining bands in the proteinase K digests just above and below the 25,700-molecular-weight markers is unknown but is of rickettsial origin because they are not seen in control lanes containing self-digested proteinase K (data not shown).

silver staining (Fig. 3). The antigenicity of these preparations was examined on a parallel immunoblot with rabbit hyperimmune anti-*C. burnetii* phase I serum. The yields and proportion of contaminating proteins and nucleic acid appear in Table 1. All three procedures appear to have extracted the phase I antigen (i.e., LPS). As shown in the silver-stained gel and Table 1, the aqueous phase of the hot phenol-water extraction contained the least contaminating protein but proportionately the most nucleic acid.

Identification of a third LPS chemotype. In comparing LPS chemotypes of *C. burnetii*, we identified an LPS type which differs from the LPSs of phases I and II. The organism of intermediate LPS type (9 mi/Cr) was recovered by M.G.P. in 1965 from the placental tissue of a female guinea pig experimentally infected 11.5 months previously with 9 mi/I. By complement fixation, this isolate was typed as phase I. Because of the tendency of phase II cells to autoagglutinate in saline, the whole-cell antigens used in many serological tests are often artificial phase II, i.e., the antigen is prepared by TCA extraction of phase I cells (14), a technique that has been used to extract LPSs from gram-negative bacteria (38).

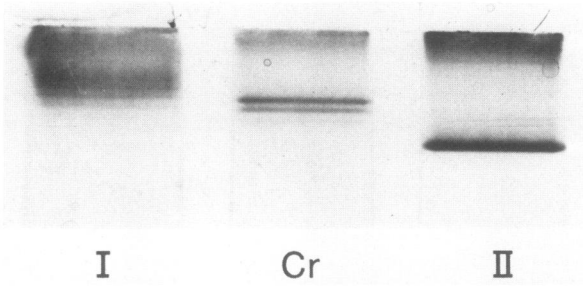

FIG. 5. Enlargement of the top portion of the polyacrylamide gel (Fig. 4B) showing more clearly the proteinase K-resistant structures which vary with LPS chemotype.

The cells after TCA extraction are used as phase II antigen. The 9 mi/Cr isolate was maintained by limited (fewer than four) passage in eggs for use in the preparation of artificial phase II antigen because it was more efficiently converted to phase II by TCA extraction. (The unusual ease of extraction of the phase I antigen from 9 mi/Cr resulted in its being known in laboratory jargon as the "crazy" variant. Hence the abbreviation Cr.) The differential efficiency of extraction of the phase I antigen, presumably LPS, from 9 mi/Cr by TCA suggested that the LPS of this isolate differs somehow. To explore this possibility, we examined the silver-stained polyacrylamide gel profiles of isolated LPS and proteinase K digests of the three variants within the Nine Mile strain (Fig. 4A and B). The LPS of 9 mi/Cr was of intermediate mobility by SDS-PAGE compared with LPSs of phase I or phase II. While the LPS of 9 mi/I appeared on 15% polyacrylamide gels as five predominant discrete bands with considerable trailing, 9 mi/Cr displayed only a single LPS species migrating at the position of the lowest of the five bands seen in phase I LPS. Phase II LPS migrated as a single band to a position in the gel lower than either 9 mi/I or 9 mi/Cr. The LPS of mi/Cr reacted, although relatively weakly, with hyperimmune rabbit antisera that recognizes the LPS of phase I *C. burnetii*, but not with antisera which recognizes the LPS of phase II (Fig. 4C and D).

Here again an unidentified component that varies between *C. burnetii* variants posessing different LPS chemotypes is seen near the top of the gel in the proteinase K-digested lysates. See enlargement in Fig. 5.

Chemical and biological properties of C. burnetii LPSs. We have preliminarily characterized some of the chemical and biological properties of the three LPS types isolated from *C. burnetii* Nine Mile (Table 2). The amount of protein contamination in the preparation was generally low. Some residual RNA contamination remained in all LPS preparations even though these preparations were treated with RNase during the purification procedure. In a few instances we were able to obtain LPS preparations with much lower (<5%) RNA contamination. On SDS-PAGE and immunoblotting these preparations behaved identically to the less pure preparations. All three LPS chemotypes contained KDO, or a KDO-like component, and heptose-like components. By weight, the phase I LPS contained the most carbohydrate. All three LPS types gelled LALs at concentrations of 0.1 ng or lower. All three *C. burnetii* LPSs were nontoxic to chicken embryos at concentrations as high as 80 μg per embryo. This is in contrast to *S. typhimurium* smooth (SCSG 225) and rough (SAI 377) LPSs, which were toxic to chicken embryos at nanogram levels.

TABLE 2. Chemical and biological comparisons of *C. burnetii* LPS with *S. typhimurium* LPSs

LPS[a]	% By weight[b]					LAL (ng)[c]	$CELD_{50}$[d]
	Protein	RNA	KDO	Heptose	Carbohydrate		
C. burnetii 9 mi/I	<1.0	11.9	0.65	1.2	44.8	≤0.1	>80 μg
C. burnetii 9 mi/Cr	2.6	15.8	0.77	2.2	35.6	≤0.1	>80 μg
C. burnetii 9 mi/II	<1.0	21.2	1.14	5.6	32.9	0.1	>80 μg
S. typhimurium SCSG 225	5.4	19.9	6.2	2.8	53.1	0.1	6 ng
S. typhimurium SAI 377	7.5	18.8	21.3	<1.0	10.4	0.1	22 ng

[a] All LPSs were prepared by the hot phenol-water extraction procedure as described in the text.
[b] Percentage dry weight. Compositional data are not corrected for protein or nucleic acid contamination. Values for each LPS type are from a single representative batch. Variation in RNA contamination between batches of *C. burnetii* LPSs prepared by this procedure was about 5 to 30%.
[c] Values are the lowest amounts resulting in LAL gel formation.
[d] $CELD_{50}$, 50% lethal dose for chicken embryos.

DISCUSSION

We extracted LPS from phase variants of *C. burnetii* and compared the isolated LPSs and *C. burnetii* cells by SDS-PAGE and immunoblotting. In the methods used here, the LPSs are the predominant components which are structurally and antigenically unique between serologically defined phase I and phase II *C. burnetii*. Although polypeptide profiles of phases I and II *C. burnetii* were similar by one-dimensional SDS-PAGE, differences in the SDS-PAGE profile were evident, particularly in the molecular-weight range of 14,400 to 25,700. This region of the gel, however, is approximately that to which the phase I LPS migrates. Thus, the minor differences in SDS-PAGE profiles in this region may be due to displacement of phase I polypeptides by comigration with the phase I LPS. Specific reagents needed to determine whether this is the case or whether there are phase-related polypeptide differences in this region are not yet available. Efforts have been made to identify immunogenic protein differences between phases of *C. burnetii* (49); we have not been able to reproduce these differences. We have chosen, however, to focus on current efforts on the LPSs of this organism because the LPS seems to be the predominant structural component that varies between phases.

The *C. burnetii* LPSs described here were found to contain a KDO-like, thiobarbituric acid reactive substance and heptose in amounts comparable to previously reported phase I and II LPS composition (2). Also as previously reported for phase I and II LPSs (2, 16), all three *C. burnetii* LPSs gelled LALs at subnanogram levels. The *C. burnetii* LPSs have been considered endotoxic (3, 34). The 50% lethal dose for chicken embryos for phase I LPS was previously determined to be 100 to 200 μg per embryo (34). In our analysis, the three *C. burnetii* LPSs were found to be nontoxic to chicken embryos at concentrations of greater than 80 μg per embryo. This is in contrast to *S. typhimurium* smooth- and rough-type LPSs which demonstrated chicken embryo lethal doses (50%) of 6 and 22 ng per embryo, respectively. The LPSs of *C. burnetii* are, therefore, over 3,000 times less toxic by weight than the *Salmonella* LPSs in the chicken embryo model.

A comparison of procedures historically used to extract the phase I antigenic component revealed that, to various degrees of efficiency and specificity, all did extract the phase I antigen identified here as LPS. It is generally believed that the phase I antigen masks the phase II antigenic determinants (3, 35) on intact *C. burnetii* cells or whole-cell antigens. Yet it is clear that antisera against *C. burnetii* in phase II, which demonstrate no phase I antibody titer by the usual serological tests of complement fixation, microagglutination, or immunofluorescence (not shown), do react with phase I components by immunoblotting (Fig. 2). Thus, once exposed after SDS-PAGE and transfer to nitrocellulose, a number of shared phase I and II proteins react with specific anti-phase II sera. Given the procedures by which phase is serologically defined, it seems reasonable to suggest that the hydrophilic LPS of phase I sterically inhibits access of antibody to the multitude of shared protein antigens of *C. burnetii*, phases I and II. Thus, the delayed appearance of serological reactivity toward phase I may be explained by the delayed appearance of antibodies against these polysaccharide antigens.

By analogy with silver-stained PAGE profiles of *Salmonella* LPS chemotypes (20), the migration of *C. burnetii* phase I LPS is similar to the laddering of smooth LPS and thus may reflect variable numbers of the equivalent of O-antigen repeat polysaccharide subunits. Phase II LPS appears to be more similar in PAGE profiles in *Salmonella* rough (Rd or Re) LPS and therefore might be expected to contain only a lipid A-KDO and truncated core oligosaccharide. The 9 mi/Cr LPS migrates on SDS-PAGE to an intermediate position as does *Salmonella* Ra or SR LPS which contains only lipid A-KDO with complete core oligosaccharide or, in the case of SR LPS, a single O-antigenic polysaccharide unit. Like the *Salmonella* LPS chemotypes (20), the chemical compositions of the *C. burnetii* LPSs shown here and elsewhere (36) are in agreement with their electrophoretic mobilities. We consider, therefore, that *C. burnetii* 9 mi/Cr LPS is an LPS with a structural complexity intermediate to the phase I and II LPSs. The data provided here are thus consistent with the hypothesis (37) that phase variation in *C. burnetii* parallels the smooth-to-rough LPS variation of gram-negative enteric bacteria.

Some additional points can be made regarding the darkly staining bands migrating near the top of the separating gel in the proteinase K-digested lysates (Fig. 2A and Fig. 5). These components were resistant to proteinase K under denaturing conditions of 1% SDS and 0.29 M 2-ME at 56°C for 1 h. The silver-stained SDS-PAGE profile of these components varied in complexity in parallel with the nontoxic LPS isolated from each of the three variants, i.e., the component from phase I appeared as a compact ladder, whereas that from 9 mi/Cr appeared as three distinct bands which migrated to a position intermediate to that of phase I and the single phase II component. These components were not recovered in the water phase after phenol extraction. The chemical composition and structure of these components are not known, but their covariation with the LPS makes it tempting to speculate that their synthesis may be coregulated or share portions of a common biosynthetic pathway with the nontoxic LPS.

On the basis of the data presented here, phase variation in *C. burnetii* might be more simply explained as mutations affecting various steps in the LPS biosynthetic pathway as occurs in other gram-negative bacteria such as *Salmonella* spp. (25) and giving rise to a spectrum of LPS chemotypes of varying complexity. The selection for phase II by laboratory passage might, according to this hypothesis, reflect the advantage of conserving the metabolic energy used to synthesize complex polysaccharides in the absence of the selective pressure for phase I imposed by an immunologically competent host. The mechanism by which the phase I LPS confers the ability to avoid or delay immune clearance needs to be examined in greater detail. A role for the phase I antigen in protective immunity is implied because Formalin-killed phase I cells are 100 to 300 times more protective than phase II cells (30). Furthermore, TCA or phenol-water extracts of phase I *C. burnetii* have been shown to elicit protective immunity against phase I challenge in mice (6).

The LPSs of phase I and II *C. burnetii* were reported to be immunologically identical (2), but this report was recently contradicted by Schramek et al. (36), who found phase I and II LPSs to be antigenically unique. In another recent report (1), reactivity of hyperimmune anti-*C. burnetii* phase I sera with phase I but not phase II LPS was described. However, phase II LPS was found by Ouchterlony immunodiffusion not to react with either phase I or phase II hyperimmune sera (1). In our studies with sensitive immunoblotting techniques in which the phase I and II LPSs are distinctly separated we demonstrated that phase I and II LPSs are antigenically unique and immunogenic on Formalin-killed whole-cell vaccines of either phase.

The unusual source from which the organism with intermediate LPS structure was recovered, i.e., the placental tissue of a chronically infected guinea pig, necessitates some discussion of possible roles of this LPS variation in the known capacity of *C. burnetii* to cause persistent and chronic infection.

Q fever is an often inapparent, nonreportable disease which, although generally benign, varies widely in severity and is capable of explosive outbreaks (23, 26, 43, 50). Recovery is generally uneventful, but in rare instances the organism persists in a chronic state and presents clinically as a culture-negative endocarditis or granulomatous hepatitis (9, 21, 42). There is also considerable evidence to suggest that persistent latent infections occur in domestic animals and humans with asymptomatic recrudescence during pregnancy (15, 24, 40, 46). The organism is shed in milk, and as many as 10^9 organisms per g of placental tissue from infected sheep and cattle have been reported (45).

It is not known whether microbial factors or some predisposition of the host leads to the rare conditions of Q fever endocarditis or granulomatous hepatitis. It is apparent that *C. burnetii* with the Cr LPS phenotype is capable of long-term survival in animals. However, two *C. burnetii* isolates recovered from the placentas of other guinea pigs in the same experiment from which 9 mi/Cr was recovered retained the phase I LPS phenotype (not shown). This LPS difference, therefore, does not appear to be a prerequisite for chronic infection. Thus, the role, if any, of this LPS variation in pathogenesis remains to be defined. Because organisms with the phase I-type LPS also appear to be capable of long-term survival in animals, the presentation of different surface components (e.g., Cr LPS) does not seem to be a requirement for the organisms to avoid immune clearance. In view of this, a more plausible explanation may be that in chronic Q fever the immune system is compromised or the organisms are sequestered so that they are not subject to the positive selective pressure for phase I by a normal immune response. Additional evidence in favor of the later possibility is that only one of four chronic Q fever endocarditis patient sera examined reacted intensely with the 9 mi/Cr LPS by immunoblot, whereas all four reacted intensely with 9 mi/I LPS (T. Hackstadt and M. G. Peacock, unpublished observations), suggesting again that transition to or selection for the Cr LPS phenotype is possible during persistent infection but not necessary. We have, however, observed the 9 mi/Cr LPS type in populations of another *C. burnetii* strain (Australian QD) that had been passed extensively in eggs (177 times) and that displayed no phase I serological reactivity (T. Hackstadt and M. G. Peacock, unpublished observations). Thus, similar mutants may have arisen independently in other strains.

The identification of an LPS type of intermediate structural complexity demonstrates that LPS variation in *C. burnetii* is more extensive than previously believed and provides suggestive evidence that virulence of *C. burnetii* may be modulated through LPS structure. The virulence and capacity of each of the three known LPS phenotypes of *C. burnetii* to establish persistent infections are the subjects of ongoing investigations.

ACKNOWLEDGMENTS

We thank Jim Kyle and Terry Brown for technical assistance and Susan Smaus for secretarial assistance. The helpful comments of John Swanson, Mark Peppler, Harlan Caldwell, Bob Anacker, and the staff of the Laboratory of Microbial Structure and Function are greatly appreciated.

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