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Steric Hindrance of Antibody Binding to Surface Proteins of *Coxiella burnetii* by Phase I Lipopolysaccharide

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The exposure of surface protein antigens on virulent phase I *Coxiella burnetii* was compared with that on avirulent phase II cells. Although anti-phase II antibodies did not bind to the surfaces of native intact phase I cells, they bound to phase I proteins if the proteins were solubilized for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by immunoblotting. In addition, removal of the phase I lipopolysaccharide (LPS) by trichloroacetic acid exposed surface proteins for reactivity with anti-phase II antibodies, as shown by immunofluorescence assays, direct antibody binding, and immunoelectron microscopy using protein A-colloidal gold conjugates. Based on these observations, a simple model of phase variation is proposed to explain the apparently conflicting notions of the identity of the phase II antigen(s). The model suggests that the phase I LPS sterically hinders access of anti-phase II antibodies to a multitude of shared protein antigens, any one of which may confer phase II specificity. Exposure of these shared protein antigens through the appearance of a more truncated LPS (phase II) or extraction of the smooth-type phase I LPS allows antibody accessibility and therefore confers apparent phase II serospecificity.

Coxiella burnetii, the etiologic agent of Q fever, is unique among obligate intracellular parasites in that it undergoes a serologically defined phase variation. Virulent phase I organisms are isolated from natural infections, whereas avirulent phase II organisms are selected for during serial laboratory passage in eggs or tissue culture (3). Determination of phase is based upon cumbersome serological techniques that rely on the reactivity of organisms with early (<20 days postvaccination) antisera. Phase II organisms bind both early and late (>20 days) antibodies. An antibody response to the carbohydrate phase I antigen is not mounted as rapidly; thus, phase I organisms are recognized only by late antisera. Only phase II antibodies are produced in response to vaccination with phase II organisms, but both phase I and II antibodies are produced in response to phase I infection or vaccination (3, 7, 8, 26). Thus, the phase II determinants are present and immunogenic on phase I organisms, although they are not available to react with phase II antibodies.

It has been believed for some time that the phase I antigen masks the phase II antigen(s), since various treatments or extraction procedures convert phase I cells to phase II reactivity (3, 6, 8, 9, 24, 32). More recent information has made it clear that the phase variation of *C. burnetii* parallels the smooth- to rough-lipopolysaccharide (LPS) transitions of gram-negative enteric bacteria (1, 12, 25). That the phase I antigen is phase I LPS is not questioned. There remain, however, numerous discrepancies in the literature regarding the nature of the phase II antigen(s). Phase-dependent differences in surface proteins or antigens have been described (31). A study with monoclonal antibodies (30) has led to the opinion that phase II specificity resides in a 29.5-kilodalton surface protein (2). However, we have reported (12) that the protein components of phase I and II cells are for the most part shared and that the unique phase-dependent antigens are the LPSs.

Here I demonstrate that the phase I LPS sterically blocks the access of antibodies to shared surface proteins and present a hypothesis of phase variation that is consistent with existing concepts and unifies some of the conflicting observations in a simple model.

MATERIALS AND METHODS

Organisms. Strain histories and propagation of the Nine Mile strain of *C. burnetii*, phase I (9mi/I), phase II (9mi/II), and an intermediate variant (9mi/Cr), have been described previously (12).

LPS isolation. LPS was extracted from *C. burnetii* by phenol-water extraction as described previously (12).

Immunoglobulins. Hyperimmune rabbit antisera against plaque-purified 9mi/I and 9mi/II were prepared by a previously described procedure (11). The immunoglobulin G (IgG) fractions from these antisera were obtained by affinity purification on protein A-Sepharose CL-4B (Sigma Chemical Co., St. Louis, Mo.) columns. Briefly, antisera were passed through a column preequilibrated with 50 mM $NaPO_4$–150 mM NaCl (PBS), and the column was washed with PBS. IgG was eluted with 20 mM citrate buffer (pH 3.6) and dialyzed overnight against two changes of cold PBS. Purified IgG was radioiodinated by the chloramine-T method (17).

Immunochemical techniques and antigens. Phase II antigens were prepared for the microagglutination assay as described by Fiset et al. (9) by trichloroacetic acid (TCA) extraction of 9mi/Cr. Phase I antigen was Formalin-killed 9mi/I. The microagglutination assay was performed as described previously (9).

The antigens for the microimmunofluorescence (MIF) assays were Formalin-killed natural 9mi/II and 9mi/I or TCA-extracted 9mi/I and 9mi/II. *C. burnetii* 9mi/I and 9mi/II were extracted with TCA by suspending the organisms at a concentration of 1 mg (dry weight) per ml of 10% (wt/vol) TCA in 0.14 M NaCl for 4 h at 4°C with occasional mixing. The cells were pelleted at 12,000 × *g* for 10 min, and the extraction was repeated. A sample was taken for assay by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The cells were then fixed with Formalin and

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used as antigens for the MIF and direct binding assays. The MIF assay was done essentially as described by Philip et al. (23), using fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin (Organon Teknika, Malvern, Pa.) as the secondary antibody.

Direct antibody binding assays were performed by mixing 7.5×10^9 organisms (about 225 μg [dry weight]) with 1.8 μCi of ^{125}I-anti-phase II antibodies in 10 mM $NaPO_4$–150 mM NaCl–3% bovine serum albumin for 1 h at 37°C. The organisms were separated from unbound antibody by centrifugation through silicon oil (d = 1.05; Aldrich Chemical Co., Inc., Milwaukee, Wis.), and the bottom of the Microfuge (Beckman Instruments, Inc., Fullerton, Calif.) tube was cut off to obtain the cell pellet for determination of bound radioactivity. Antigens for this assay were the same as those used for the MIF assay described above.

Fluorescence spectroscopy. A 100-μg (dry weight) portion of TCA-extracted 9mi/I or nonextracted 9mi/I was incubated with anti-9mi/I IgG (1:250) in PBS for 30 min at room temperature with constant rotation. The organisms were pelleted and washed with PBS in a Beckman Microfuge 12. The IgG-coated organisms were then incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin (Organon Teknika) for 30 min at room temperature and washed twice with PBS. The cells were resuspended in PBS, and fluorescence at 519 nm was determined in a Varian SF330 spectrofluorometer (Varian, Palo Alto, Calif.) using an excitation wavelength of 450 nm.

Immunoelectron microscopy. Antigens for protein A-colloidal gold localization of bound antibody were those described above for the immunofluorescence and direct antibody binding assays. The procedures for protein A-colloidal gold staining have been described (4).

SDS-PAGE and immunoblotting. The procedures for SDS-PAGE and immunoblotting have been described previously (13).

Radioimmunoprecipitation. Phase I and II *C. burnetii* cells were radioiodinated by the lactoperoxidase procedure as previously described (12). The organisms were solubilized in a lysis buffer (33) of 0.5 M Tris hydrochloride (pH 7.4)–0.15 M NaCl–0.01 M EDTA–0.5% (wt/vol) deoxycholate–0.5% (vol/vol) Triton X-100–0.1% (wt/vol) SDS for 1 h at 37°C with constant rotation and clarified by centrifugation at 100,000 × g for 30 min. For immunoprecipitation, 1 ml of lysate was incubated with 200 μl of IgG for 1 h at room temperature with rotation. Protein A-Sepharose CL-4B was added, and the mixture was incubated for an additional hour at room temperature. The precipitates were pelleted in a Beckman Microfuge 12 and washed three times with lysis buffer and once with PBS. The samples were solubilized for SDS-PAGE by boiling for 5 min in 62.5 mM Tris hydrochloride (pH 6.8)–2% SDS–4% 2-mercaptoethanol–10% glycerol.

RESULTS

Specificity of the purified immunoglobulins. The specificity of the IgGs was determined by agglutination, immunofluorescence, immunoblot, and immunoprecipitation assays. The anti-phase II antibodies did not bind to the surfaces of intact phase I cells, as assessed by microagglutination or MIF, but reacted well with the phase II antigens. Anti-phase I IgG bound both the phase I and II antigens in these assays (Table 1). The same antibodies were examined by immunoblotting (Fig. 1A and B). As previously shown with other, crude antisera (12), the phase II antibodies reacted equally well with both phase I and II proteins once these proteins were solubilized and exposed by SDS-PAGE and transfer to nitrocellulose. The most prominent protein antigens had molecular masses of approximately 23 and 48 kilodaltons. Several other minor antigens, also shared, were visible upon longer exposure. The proteins recognized by the anti-phase I antibodies were also shared between both phases. That the shared antigens are proteins is demonstrated by their susceptibility to protease digestion (12). The unique phase-dependent antigens are the LPSs.

Anti-phase II antibodies were radioiodinated by the chloramine-T method (17), and the specificity of the ^{125}I-antibodies was also assayed by immunoblotting (Fig. 1C). Although the reactivity of the antibodies to the predominant protein determinants of phase I and II cells was retained, the antibodies to the phase II LPS were apparently inactivated by the iodination procedure.

Radioimmunoprecipitation assays similarly detected few differences between recognized antigens for the two phases (Fig. 1D). In general, any antigen precipitated from one phase was detected on the other. The only exception was a high-molecular-mass component precipitated from phase II cells by anti-phase II IgG. This component may be related to the proteinase-K-resistant component, previously described, that varied between phases (12).

Extraction of the phase I antigen. TCA extraction is known to convert phase I *C. burnetii* cells to phase II reactivity (6, 8, 9, 24, 33). TCA has been used to extract enterobacterial LPSs (5), and the extraction of *C. burnetii* phase I antigen (LPS) has been verified (12). The extraction of the phase I LPS is demonstrated above (Fig. 1A). Phase II LPS was not extracted under the same conditions (Fig. 1B), and this treatment had little effect on the protein determinants.

Exposure of the phase II antigenic determinants. Anti-phase II antibodies, which did not react with the formalinized native surfaces of intact phase I cells, bound phase I organisms after TCA extraction and exposure of shared phase I and II surface proteins, as demonstrated by immunofluorescence assays (Table 1). TCA extraction did not alter the reactivity of anti-phase I antibodies with either phase I or II cellular antigens nor did it affect the interaction of anti-phase II antibodies with phase II cells. Despite the extraction of phase I LPS by TCA, enough LPS remained associated with the cells (Fig. 1A) so that the endpoint of fluorescence with anti-9mi/I IgG was unchanged. The reduced amount of LPS on TCA-extracted phase I cells was manifested by decreased brightness of fluorescence. Fluorescence spectroscopy revealed only about a twofold decrease in binding of anti-9mi/I IgG to TCA-extracted 9mi/I cells.

Similarly, radioiodinated anti-phase II antibodies displayed minimal binding to phase I cells, but the amount of antibody bound increased by 10-fold after TCA extraction of

TABLE 1. Specificity of anti-9mi/I and anti-9mi/II antibodies and effect of TCA extraction on the surface exposure ofshared phase I and II antigenic proteins

Antiserum[a]	Agglutination[b] with antigen:		Immunofluorescence[b] with antigen:			
	I	II	I	I-TCA[c]	II	II-TCA[c]
Anti-9mi/I	256	128	4,096	4,096	512	512
Anti-9mi/II	<4	512	<16	1,024	4,096	4,096

[a] IgG fraction of hyperimmune rabbit antisera.
[b] Reciprocal of highest dilution giving a positive reaction.
[c] Particulate antigen after TCA extraction as described in the text.

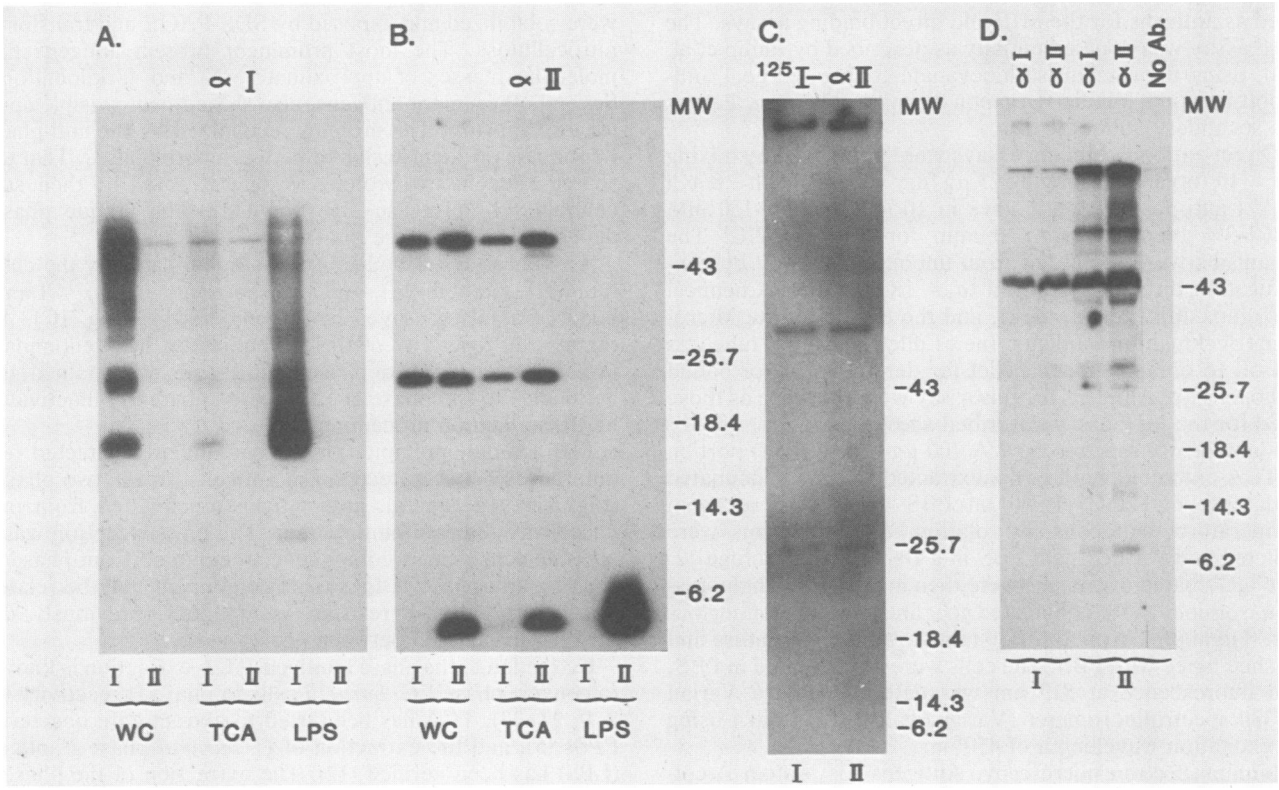

FIG. 1. Immunoblot analysis of the specificity of anti-phase I (A) and anti-phase II (B) IgGs. Whole-cell lysates of 9mi/I and 9mi/II (WC), whole-cell lysates of TCA-extracted 9mi/I and 9mi/II, and purified LPSs of 9mi/I and 9mi/II were used. (C) Reactivity of ^{125}I-anti-phase II antibodies with whole-cell lysates of 9mi/I and 9mi/II subjected to SDS-PAGE on a 10% acrylamide gel. (D) Autoradiogram of surface-iodinated phase I or II cells immunoprecipitated by anti-phase I (αI) or anti-phase II (αII) IgGs. The far right lane is a control with no antibody added. MW, Molecular weight (molecular weights shown in thousands).

the phase I LPS (Table 2). Since the activity of the anti-phase II LPS antibodies was inhibited by the radioiodination procedure and only antibodies to protein antigens remained active (Fig. 1C), the antibody binding to TCA-extracted phase I cells resulted from exposure of shared phase I and II surface proteins. The amount of antibody bound by native or TCA-extracted phase II cells was greater, but this may have been due to an incomplete extraction of the phase I LPS (Fig. 1A).

Immunoelectron microscopy. The binding of anti-phase II antibodies to the surfaces of native and TCA-extracted phase I and II cells was further studied by protein A-colloidal gold immunoelectron microscopy. Anti-phase II antibodies were not detected on the surfaces of native phase I cells but bound to TCA-extracted phase I cells (Fig. 2A and B). As indicated above, anti-phase II antibodies bound both native and extracted phase II cells (Fig. 2C and D).

DISCUSSION

It has been believed for some time that the carbohydrate phase I antigen of *C. burnetii* masks the phase II antigenic determinant(s) (3, 6, 8, 9, 24). There are two possible interpretations of the masking phenomenon dependent on the identity of the phase II antigen(s). (i) The addition of phase I sugars may block the antigenic determinant of phase II LPS, or (ii) the phase I LPS may sterically block the access of antibodies to shared phase I and II surface proteins. The latter possibility seems more likely for the reasons discussed below.

Substitution of enterobacterial rough LPSs with additional carbohydrate moieties sterically blocks the antigenicity and immunogenicity of core LPS structures, and the immunodominant components are the terminal sugars (19). The immunochemical properties of *C. burnetii* LPSs seem to be similar. For example, anti-lipid A or anti-phase II LPS antibodies do not recognize *C. burnetii* phase I LPS even though lipid A and presumably phase II structural components form a core for the addition of the phase I carbohydrates (12; T. Hackstadt, unpublished observations). It is unlikely that exposure of the phase II LPS determinant by TCA extraction explains changes in serological reactivity of phase I cells since TCA extraction removes the phase I LPS and does not modify it to phase II LPS. Furthermore, phase I infection or vaccination stimulates the production of anti-phase II antibodies, although these antibodies are not di-

TABLE 2. Binding of ^{125}I-phase II antibodies to intact and TCA-extracted *C. burnetii*

Antigen	Radioactivity (cpm) bound ($\bar{x}$ ± SEM)
9mi/I	16,457 ± 1,040
9mi/I-TCA[a]	166,025 ± 1,440
9mi/II	729,287 ± 63,486
9mi/II-TCA[a]	524,971 ± 127,471

[a] TCA-extracted cells.

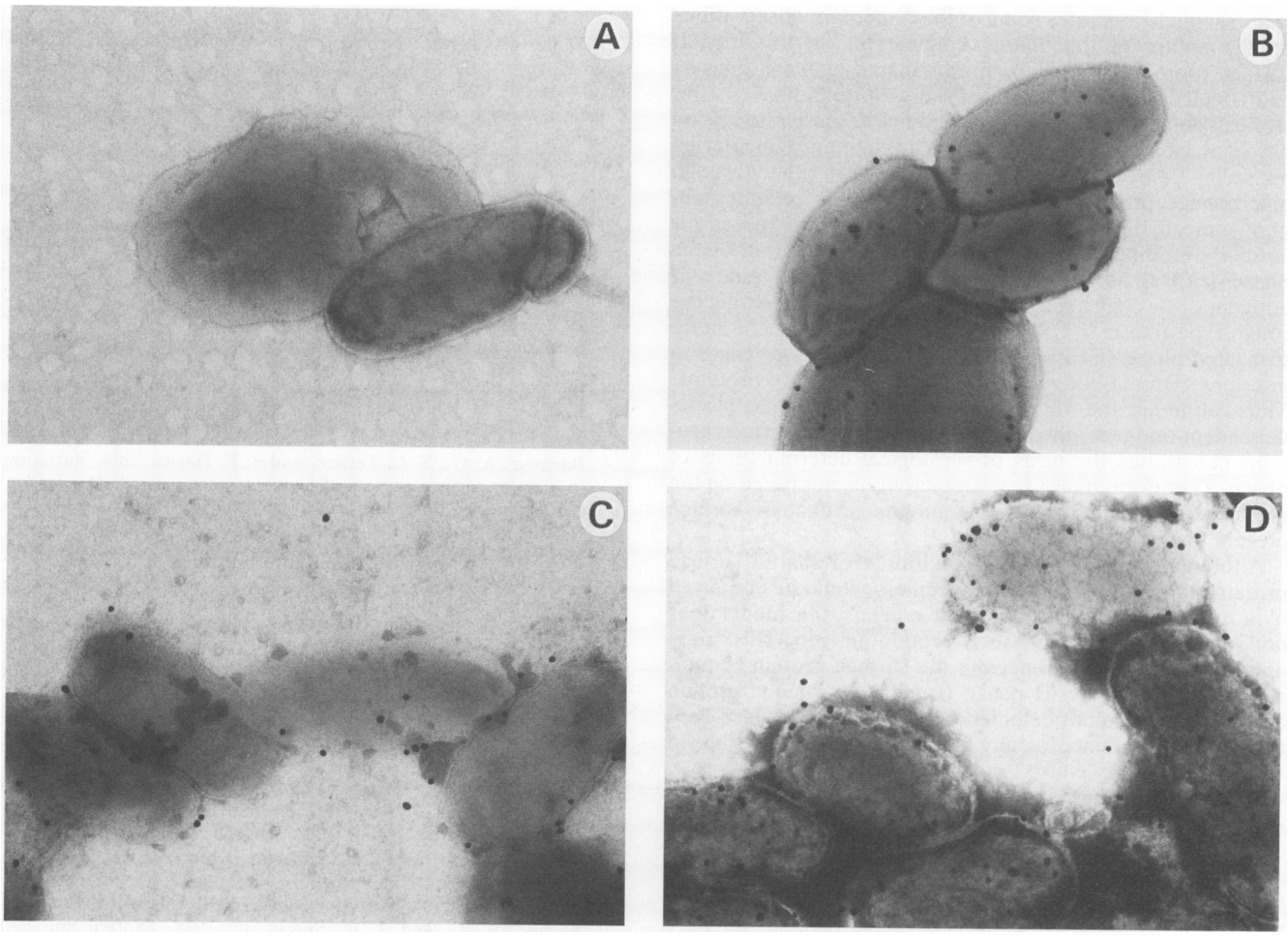

FIG. 2. Protein A-colloidal gold immunoelectron microscopy of anti-phase II IgG binding to native 9mi/I (A), TCA-extracted 9mi/I (B), native 9mi/II (C), and TCA-extracted 9mi/II (D).

rected at the unique antigenic determinant on phase II cells, the LPS (7, 8, 12, 20, 26).

A more likely explanation is that the phase I LPS, with its extended carbohydrate structure, sterically blocks the access of antibody to surface proteins. The *C. burnetii* surface proteins so far examined are not accessible to antibody on native phase I cells but become exposed after extraction of the phase I LPS. The exposure of shared phase I and II surface proteins was demonstrated by direct binding of ^{125}I-anti-phase II protein antibodies to TCA-extracted phase I cells, as well as the increased reactivity of these extracted phase I cells with anti-phase II antibodies in immunofluorescence assays and immunoelectron microscopy studies. Similarly, Williams et al. (30) described a monoclonal antibody that recognized a 29.5-kilodalton protein of phase II but not native phase I *C. burnetii* and subsequently interpreted this as indicating that phase II specificity resided in that protein (2). Interestingly though, the protein could be exposed on phase I cells by chloroform-methanol extraction. This may represent another example of exposure of a protein antigen by modification of the native phase I surface. Analogous steric hindrance of antibody binding by LPS has been described for the PhoE pore protein of *Escherichia coli*, in which the protein is inaccessible on cells possessing smooth LPS (27, 28).

Based on these observations, I propose a simple model

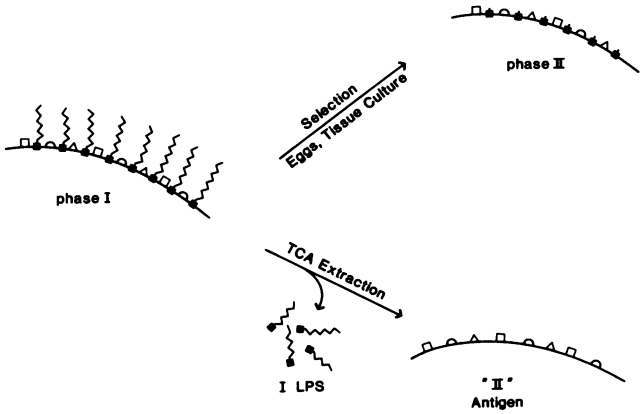

FIG. 3. Model of *C. burnetii* phase variation. In this model, the shared phase I and II surface proteins are depicted as open symbols on the exterior surface of the outer membrane. On native phase I cells, the phase I LPS is believed to sterically inhibit the access of antibodies to the surface proteins. Phase II seroreactivity is conferred by exposure of these proteins either by chemical extraction of the phase I LPS or by selection for a more truncated LPS chemotype. Here the phase II LPS is depicted as a closed box (representing a lipid A core) with only a short carbohydrate extension. No particular surface protein is believed to be a unique phase II determinant as accessibility of any surface protein antigen would confer phase II serospecificity.

(Fig. 3) which integrates some of the discordant observations on the nature of the phase II antigen(s). In this model, surface proteins of *C. burnetii* are shared between phase I and II cells, but the presence of the wild-type, phase I LPS sterically blocks the access of antibodies to these proteins on native phase I cells. These proteins are immunogenic during phase I *C. burnetii* infection or vaccination either through macrophage processing of killed *C. burnetii* or other unknown mechanisms. These surface proteins are exposed for antibody binding either through chemical extraction of the phase I LPS, as is done to create "artificial" phase II antigen, or through selection of phase II mutants bearing the rough-type phase II LPS. In the latter instance, the more truncated phase II LPS would not be of sufficient length to interfere with antibody accessibility to the surface proteins. Thus, although the LPSs appear to be the unique phase-dependent antigens, the accessibility of shared surface proteins constitutes the basis of serological determinations of phase. Therefore, phase II seroreactivity is not based on the presence of phase II LPS but on the artifact of surface protein exposure.

Although there are likely a multitude of shared surface protein antigens, phase II serospecificity could be conferred by a response to any surface protein. Thus, this model does not suggest a specific phase II protein antigen. This study does not rule out differences in the surface protein components of phase I versus phase II cells, but unique protein components were not detected by the techniques used here nor is there evidence of any surface protein accessible to antibody on intact phase I cells.

If this model is correct, it would further suggest that surface proteins may not be promising vaccine candidates since they are not exposed to react with antibody and all clinical isolates of *C. burnetii*, whether from acute Q fever or Q fever endocarditis, possess a phase I LPS (11). It is known that phase II cells are much less effective vaccines than are phase I cells (22). Roles for both antibody (16) and cell-mediated immunity (15, 18) as protective mechanisms in Q fever have been proposed. Although the accessibility of surface components to T-cell recognition sites was not examined here, it seems likely that if antibody cannot reach the surfaces of the microbes, neither would immune cells be able to contact *C. burnetii* surface proteins.

In addition to shielding *C. burnetii* from components of the immune system of the host, the wild-type LPS may also provide an effective barrier to other deleterious substances. In numerous experiments in which phase I and II *C. burnetii* cells were labeled with ^{125}I by either the lactoperoxidase (21) or Iodogen (10) procedures, phase II organisms were consistently labeled 5 to 10 times more efficiently than phase I cells (T. Hackstadt, unpublished observation). It seems plausible that the phase I LPS may help protect the cell surface proteins from chemical modification by short-lived small molecules, such as reactive oxygen intermediates or halides, that either do not penetrate the LPS barrier efficiently or are enzymatically generated at such a distance from the outer membrane that they lose activity by the time they diffuse to the surface. Like that of other gram-negative bacteria (29), the resistance of *C. burnetii* to host bactericidal systems may be related to the length of the polysaccharide component of its LPS.

C. burnetii has clearly evolved a number of survival mechanisms to allow its growth within the harsh environment of the phagolysosome (14). The phase I LPS likely represents another means of providing for the survival of phase I organisms in the face of host defenses.

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