

Antigenic Structure of *Coxiella burnetii*

A Comparison of Lipopolysaccharide and Protein Antigens as Vaccines against Q Fever

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INTRODUCTION

Immunization with inactivated phase I *Coxiella burnetii* whole cells protects animals and humans against experimental and natural Q fever.^{1,2} The characterization of *C. burnetii* membrane antigens that play a role in the development of immunity revealed phase I lipopolysaccharide (LPSI) and a large number of proteins as possible vaccine candidates.^{3,4} A new vaccine prepared from the residue of chloroform-methanol-extracted phase I cells (CMRI) is efficacious in animals.⁵ However, immunization with carrier-linked, recombinant DNA-produced or synthetic peptide subunit vaccines derived from protective epitopes of *C. burnetii* is highly desirable. Alternatives to the protein-based strategies include the use of the *C. burnetii* non-toxic LPSI to provide efficacious immunomodulation and protective epitopes. The studies described here may facilitate the identification of protective protein or LPSI epitopes as target(s) for Q fever vaccine development.

Another approach for vaccine development against Q fever is the consideration of antigenic differences among the morphological cell types of *C. burnetii*.⁶ In recent studies, the developmental cycle of *C. burnetii* was shown to consist of transverse binary fission and sporulation.^{7,8} The obvious ultrastructural differences among the morphological cell types (large and small cells) are correlated with the expression of developmentally regulated antigenic variation.⁶ Both LPSI and protein antigenic differences were demonstrated among the cell types by post-embedding immunoelectron microscopy and immunoblotting. Murine monoclonal antibodies against the LPSI labeled the cell walls of the small cells and a subpopulation of the highly organized large cells. The loosely organized large cells, apparently either cells in the death-and-decline phase of growth or deteriorating mother cells, were not labeled by monoclonal antibodies against the LPSI. The endogenous spore was also not labeled by the anti-LPSI monoclonal antibodies.

A gene encoding a 29.5-kDa protein (P1) is turned on as part of the program of differential gene expression during germination and growth of the small "resting

370

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cell," the end product of sporulation.⁶ P1 is an immunodominant protein expressed primarily on the cell wall of the intermediate-to-large cells of *C. burnetii*. Inferentially, the protected epitopes of the spore and a subpopulation of the small cells go unrecognized, since they were not detected by hyperimmune sera against the whole cells, the cell walls, or the peptidoglycan-protein complex.⁶ Thus, the spore and the small resting cells may evade the immune response by antigenic variation. These latter two forms of the microorganism are likely to initiate the human infection by being inhaled or being inoculated by ticks. Currently we know nothing about the ability of these "covert" cells to induce an infection or immune response in humans. In experimental laboratory animals the pressure-resistant small cells of *C. burnetii* are prepared and used as the infectious challenge agent by either aerosol or intraperitoneal injections to test the efficacy of potential vaccines.

The current level of protection against infection by *C. burnetii* after vaccination of animals and humans with whole cells is inadequate because of (i) the potential for immunopathological damage, (ii) the partial protection which does not adequately restrict growth of the microorganism in the phagolysosome, and (iii) the elicitation of immune responses against several immunodominant non-protective antigens. Because the spore and a subpopulation of the small cells of the *C. burnetii* developmental cycle probably evade host immunity through antigenic variation, there is a need for an efficacious subunit vaccine prepared from the spore and small cells.

RESULTS

LPSI as Vaccine and Diagnostic Reagent

The LPSI of *C. burnetii* is a virulence determinant which provides a barrier against the combined effects of the immune response. The infection of guinea pigs by phase I cells produces an early LPSI-antibody immune complex,⁹ followed by high antibody titers to LPSI late in the infection.¹⁰ In infected humans the anti-LPSI antibody response occurs very late in the course of the illness, with the induction of high titers in chronic Q fever (J. C. Williams and T. J. Marrie, unpublished results). Since the LPSI is 100 to 1000 times less endotoxic than the LPSs of *Escherichia coli* and *Salmonella typhimurium*,¹¹ it should be a good vaccine, providing immuno-enhancement and protective immunity.

The immunogenicity of purified LPSI was tested in mice by intraperitoneal (i.p.) injection. Immunization of mice with 100 μ g of LPSI suspended in saline, without an adjuvant, induced significant antibody responses against LPSI and phase I and phase II whole cells (TABLE 1). Also, an increase in the cellular immune response was generated as a result of the injection of LPSI (TABLE 1). Lymphocytes from immunized animals responded to the T cell mitogen, concanavalin A, and to recall antigens more vigorously than did saline-injected controls.

Immunized animals challenged with aerosols of the small cells of *C. burnetii* survived with only about a 0.8-log reduction of infectious microorganisms per milligram of spleen (TABLE 2). Ninety percent of the unimmunized animals were killed by the aerosol challenge. Challenge by the i.p. route killed 80% of the animals in the unimmunized group but only 30% of the group immunized with LPSI. The immunized group was more effective than the unimmunized group in

TABLE 1. Immune Response of LPSI-Vaccinated A/J Mice

Component Injected ^a	Antibody Titers (ELISA)			Lymphocyte Responses ^b			
	LPSI	Phase I	Phase II	Con A	WCI	CMRI	LPSI
PBS	<20	<40	<40	40	3.5	5.9	2.3
LPSI	160	4960	3840	70	5.4	12.0	3.6

^a Mice were injected i.p. with 100 μ g of LPSI (phase I purified lipopolysaccharide) or saline (PBS) alone 21 days before responses were measured.

^b Stimulation index of lymphocytes. Con A, concanavalin A; WCI, phase I whole cells; CMRI, phase I chloroform-methanol-extracted residue.

reducing the replication of *C. burnetii*. The immunized group reduced the infectious burden by 2.3 logs per milligram of spleen.

The pathogenesis of Q fever was notably different in animals challenged by the aerosol and the i.p. routes. The small differences in spleen weight observed with aerosol challenge were not characteristic of the i.p. challenge route (TABLE 2). Spleen weights were 8.4 times larger in the i.p. challenged group than in the aerosol group. Three logs more infectious *C. burnetii* were in the spleens of control animals challenged by the i.p. rather than by the aerosol route. Calculations based on the number of microorganisms per milligram of spleen showed 5.7 times more *C. burnetii* in the i.p. challenged animals. Therefore, the induction of pathogenic mechanisms by aerosols of *C. burnetii* was obviously different from those induced with challenge by the i.p. route.

The immunized and challenged animals showed no overt signs of illness, even though their spleens contained about one million infectious microorganisms. Thus, protective efficacy measured by live versus dead animals was an inadequate measure of protection. The LPSI elicited an immune response which reduced the replication of *C. burnetii* in mice challenged by the i.p. route but not in those challenged by the aerosol route. The fact that immunized animals were protected from the lethal effects of aerosol challenge without a significant reduction in the replication of *C. burnetii* in the spleens indicates that another host organ may be a target of the lethal effects of Q fever after aerosol challenge.

TABLE 2. Pathogenic Reactions of LPSI-Vaccinated Mice Challenged with *C. burnetii*

Component Injected ^a	Aerosol Challenge ^b				i.p. Challenge			
	No. Deaths/ No. Tested	MTD ^d	Organisms/ Spleen ^c		No. Deaths/ No. Tested	MTD ^d	Organisms/ Spleen ^c	
			log ₁₀	Wt (mg)			log ₁₀	Wt (mg)
PBS	9/10	7.1	6.5	94	8/10	4.9	9.5	790
LPSI	0/10	—	5.6	80	3/10	9.6	6.9	410

^a 100 μ g of LPSI or saline (PBS) alone was given i.p.

^b Mice received 10 LD₅₀ of phase I, Henzerling strain, *C. burnetii* 21 days after vaccination.

^c Infectious organisms per spleen (log₁₀) and spleen weight (Wt) were measured 7 days after challenge.

^d MTD, mean time to death.

*Protein Antigens as Vaccines and Diagnostic Reagents**The 62-kDa Heat-Shock Protein*

In another study, an operon encoding a "common antigen" of *C. burnetii* was cloned and sequenced.¹² This 62-kDa protein of *C. burnetii* is highly homologous with a conserved protein of other prokaryotes and eukaryotes. The 62-kDa protein was purified by preparative SDS-polyacrylamide gel electrophoresis and tested as a vaccine candidate in the A/J mouse model (TABLE 3). The protein antigen was effective in eliciting a humoral immune response to itself and to phase II whole cells without the induction of antibody against phase I whole cells. This indicates that the 62-kDa protein is abundantly distributed on the surface of phase II but not phase I cells. Lymphocytes from C57BL10/ScN mice immunized i.p. with 100 µg of phase I cells or CMRI were tested 14 days post-vaccination for their response to the purified 62-kDa protein. The 62-kDa protein was neither mitogenic nor effective as a recall antigen (data not shown). However, lymphocytes from BALB/c mice immunized i.p. with 100 µg of the 62-kDa protein at weekly intervals for 7 weeks responded with high levels of antibodies against the

TABLE 3. Response of A/J Mice to the 62-kDa *C. burnetii* Protein

Component Injected ^a	Antibody Titer to Test Antigen ^b		
	62 kDa	Phase I	Phase II
PBS	<80	<80	<80
62 kDa	5120	<80	2560
62 kDa/Al(OH) ₃	10240	<80	2560

^a Mice were injected i.p. with 100 µg protein (62 kDa) purified from recombinant *E. coli* (pCS26C1),¹² with Al(OH)₃-precipitated protein, or with saline (PBS).

^b Specific antibody titers (21 days post-immunization) were determined by ELISA.¹⁰ Phase I, phase II, phase I and phase II whole cells, respectively.

62-kDa protein and 2.4 times greater lymphocyte activity against recall antigen than did control lymphocytes. Although the 62-kDa protein was both antigenic and immunogenic, we predict that the 62-kDa protein may not be a protective antigen against phase I infections because it is not located on the cell surface.

The diagnostic utility of this protein lies in the fact that animals and humans mount an antibody response against the 62-kDa protein. Analysis by immunoblot of acute, convalescent, and chronic Q fever patients' sera showed a significant rise in antibody to the 62-kDa protein (data not shown). Certain *Coxiella*-specific peptides may be good candidates for the development of peptide-based diagnostic reagents. Because of the immunogenicity of the 62-kDa protein, it may be appropriate for the detection of anti-peptide antibody occurring during convalescence or chronic Q fever progression.

The P1 Surface Protein

A major surface antigen of roughly 29.5 kDa (P1) was purified to homogeneity (N. Banerjee-Bhatnagar *et al.*, manuscript in preparation) and tested as a poten-

TABLE 4. Moderation of *C. burnetii* Infection in Mice Injected with *C. burnetii* LPSI or P1

Component Injected ^a	Prechallenge Antibody Titer to Test Antigen ^b			Post-Challenge ^c	
	LPSI	Phase I	Phase II	Spleen Wt. (mg)	Organisms/Spleen ^d (log ₁₀)
PBS	<40	<40	<40	913	9.2
LPSI	400	800	<40	708	7.5
P1	25600	12800	25600	502	6.6

^a Mice received two 25- μ g injections i.p. of indicated antigen or saline (PBS) at 14-day intervals.

^b Measured by ELISA 14 days after the final injection. Phase I, Phase II, phase I and phase II whole cells, respectively.

^c Mice were injected i.p. with 10⁸ phase I *C. burnetii* 28 days after the second injection.

^d The number of infectious microorganisms per spleen was measured 7 days after challenge.

tial vaccine (TABLE 4). The P1 preparation had 0.1% 3-deoxy-D-manno-octulosonic acid (KDO)¹¹ and undetectable LPSI epitopes by monoclonal antibodies. The purified protein was mitogenic for normal murine lymphocytes, producing a stimulation index of 2.1. The i.p. injection of P1 induced significant levels of antibodies against purified LPSI, phase I and phase II whole cells (TABLE 4). The induction of antibodies against purified LPSI after the injection of P1 may be explained by either the presence in the P1 preparation of residual LPSI which copurified with P1 or the presence of peptides which copurify with the LPSI. The amino acid composition of highly purified LPSI indicates that peptides are present in the preparation (TABLE 5). Further studies indicated that peptides in the LPSI fraction may not be immunogenic at low concentrations, as antibodies against phase II whole cells were not detected after two 25- μ g injections of purified LPSI.

TABLE 5. Amino Acid Composition of Purified LPSI

Amino Acid	Amount ^a (nmol/mg LPSI)
Asp	26.65
Thr	4.60
Ser	11.60
Glu	163.51
Gln	ND
Pro	5.75
Gly	12.86
Ala	3.50
Val	4.31
Cys	2.22
Met	0.73
Ile	1.65
Leu	2.73
Tyr	2.55
Phe	1.10
Trp	ND
Lys	6.45
His	UD
Arg	4.26

^a ND, not done; UD, undetectable.

The protective efficacy of P1 was greater than that of LPSI alone (TABLE 4). After i.p. challenge, the spleen weight was reduced to 77% and 55% of that of controls by previous immunization with LPSI and P1, respectively. P1 was more efficacious than LPSI in reducing the number of infectious microorganisms per milligram of spleen. Although LPSI and P1 induce significant protection against challenge, P1 induced a more vigorous protective immune response. This is the first indication that a purified protein may be more efficacious than either LPSI or phase I whole cells.

The P2 Surface Protein

The gene encoding another surface protein (P2) was detected by monoclonal antibodies against P1 in a genomic library of *C. burnetii*.¹³ Although the P2 protein

TABLE 6. Immunological Responses of Mice to P2 Peptides

Peptide ^a	No. of Amino Acids	Anti-peptide Activity of Test Serum ^b			Activity of Anti-peptide Serum with Phase II ^c	Mitogenic Activity ^d
		Normal	Hyperimmune	Anti-peptide		
A	20	—	+	+	+	—
B	20	—	+	+	—	—
C	18	—	+	+	—	—
D	16	—	—	+	—	—
E	18	—	—	—	—	—

^a Synthetic peptides prepared from the deduced amino acid sequence of the cloned P2 gene. Peptide A is the amino-terminal and peptide E is the carboxyl-terminal peptide of the sequence.

^b Activity of indicated serum was tested in an ELISA with the indicated peptide adsorbed to the polystyrene plate. The hyperimmune serum was derived from mice infected with Nine Mile phase I, clone 7; anti-peptide sera were obtained from mice injected with the indicated peptide in incomplete Freund's adjuvant.

^c Exposure of the peptides on the surface of Nine Mile phase II, clone 4, was tested in an ELISA with anti-peptide sera obtained by immunization with the indicated peptide.

^d Mitogenic activity of each peptide was tested on normal spleen cells of mice.

was first identified by P1 monoclonal antibodies, the proteins were obviously different. The amino acid composition of the purified P1 (C. Snyder, personal communication) was not the same as the predicted amino acid composition of the P2 gene product.¹³ Thus, P1 and P2 share similar epitopes recognized by monoclonal antibodies.

The recombinant *E. coli* carrying the gene encoding P2 did not produce an intact protein. The synthetic peptide approach¹⁴ was adopted for the preparation of 5 peptides along the length of P2. The method of Jameson and Wolf¹⁵ was chosen to predict which regions of the protein would be antigenic. This algorithm integrates values of hydrophilicity, surface probability, backbone flexibility, and the secondary structure predictions of Chou and Fasman¹⁶ and Garnier *et al.*¹⁷ in order to calculate an antigenic index. The peptides (TABLE 6) were injected i.p. into mice after suspension in either saline, alum [Al(OH)₃], or incomplete Freund's adjuvant.¹⁸ While none of the peptides reacted with normal mouse serum, peptides A, B, and C were antigenic when reacted with serum obtained from

animals infected with phase I *C. burnetii* (TABLE 6). Peptides A through D were immunogenic when prepared in an emulsion of incomplete Freund's adjuvant. Peptide A was the only peptide which elicited antibody that gave a reaction against the surface of phase II cells. None of the peptides were mitogenic for normal spleen cells. We were not able to determine the apparent molecular size of the P2 gene product, because the anti-peptide sera did not react with native antigens of *C. burnetii* in an immunoblot assay (data not shown). Thus, we will have to chemically couple the peptides to different carriers to obtain antibodies which react with native protein antigens.^{19,20}

DISCUSSION

Our current strategy for the development of molecular vaccines against Q fever centers around the identification and characterization of both immunodominant and non-immunodominant antigens. The development of an efficacious vaccine against *C. burnetii* infection has been only marginally successful because of the highly reactogenic properties of the phase I whole cell immunomodulatory complex (IMC).²¹ The inactivation of the IMC by chloroform-methanol extraction of phase I whole cells has resulted in the development of an efficacious non-reactogenic CMRI vaccine.⁵ The antigenic components required for the development of an efficacious Q fever vaccine can be more easily identified now that we have developed ways to reduce the reactogenic properties of whole cells.

Impediments to the development of Q fever vaccines are more easily identified now that the components of the IMC are known.²¹ Since the activity of the IMC is not directly related to the LPS of *C. burnetii*, the LPS may be considered as an efficacious biological response modifier.^{5,11} The undesirable side effects of endotoxin usually preclude the use of the whole molecule in the strategy for vaccine development.²² For the inclusion of LPS in protein- or peptide-based vaccines, we need to take advantage of the beneficial effects of synthetic or detoxified endotoxins.²³ Since the LPS of *C. burnetii* is a very poor endotoxin,¹¹ we are testing the safety, immunogenicity, and efficacy of the phase I LPS in animal models. Our results were encouraging, since LPSI was both immunogenic and efficacious. Immunized mice resisted lethal challenge doses, and there was a reduction in the number of infectious phase I *C. burnetii* in the spleens of challenged mice. The safety of the *C. burnetii* LPS in humans has not been evaluated.

The antigenic structure of the different morphologic forms of *C. burnetii* is very complex.⁶ A recent finding that the expression of the major 62-kDa protein antigen is regulated by a heat-shock promoter has encouraged a renewed effort to understand the physiology and genetic regulatory mechanisms controlling the survival of *C. burnetii* in the phagolysosome.²⁴ The 62-kDa protein is associated with the cell wall,¹² and it is apparently released from the cell into the phagolysosome by an unknown mechanism.²⁴ Although the protein is abundantly exposed on the surface of phase II cells, specific polyclonal antibodies failed to detect the protein on the surface of phase I cells. This may be due to a close association of the protein with other molecules which sterically hinder the binding of polyclonal antibodies. Alternatively, since polyclonal antibodies were developed against denatured protein, they may not recognize native conformational epitopes on the surface of cells.

The finding of mammalian homologs²⁵ of the "common antigen," which is the major antigenic protein of a wide variety of pathogenic bacteria²⁶ and of *C. burnetii*,¹² is an example of molecular mimicry.²⁷ The shared common antigenic de-

terminants of mammalian and bacterial origin are targets of immune responses which may induce autoimmune reactions. The T cell-reactive epitope in the 65-kDa protein of *Mycobacterium leprae*, *M. tuberculosis*, and *M. bovis* is predicted to play a major role in the development of autoimmunity.²⁸ Although such an immunoreactive epitope has not been identified in the *C. burnetii* 62-kDa antigen, there may be other epitopes of equal importance in the induction of chronic forms of Q fever.

Protein-based subunit vaccines against *C. burnetii* infections are highly desirable alternatives to the whole-cell vaccines. Determinants of the P1 protein were exposed on the surface of both phase I and phase II cells. Much to our surprise, P1 was absent in the spore and a subpopulation of the cell walls of some of the small cells.²⁹ The presence of P1 in only the large cells indicates that the expression of the P1 gene is developmentally regulated. These results indicate that continuous or chronic infection by *C. burnetii* may be perpetuated by the inability of the host immune system to recognize the spore and a subpopulation of the small cells. Recently, we have discovered that the determinants of spores and a subpopulation of small cells are not detected by antibodies obtained from infected or immunized humans (unpublished results) and animals.⁶ Although P1 thus may not ultimately be suitable for use in a protein-based vaccine, it will probably serve as a source of peptides or DNA probes for diagnostics.

The preparation of monoclonal antibodies against P1 led to the identification of P2. P2 is related to P1 only because it carries a cross-reactive epitope recognized by monoclonal antibodies.¹³ Since the amino acid compositions of these two proteins were significantly different, the monoclonal antibody specificity probably resided in the structural specificity of modified amino acids. Four of the five synthetic peptides derived from the deduced nucleotide sequence of the P2 DNA were sufficiently immunogenic to induce an anti-peptide antibody response. While three of the peptides were antigenic, none of them were mitogenic. The predicted surface orientation of peptide A was verified by anti-peptide A antibodies, because they recognized the surface of phase II cells. All of these responses of the synthetic peptides were elicited in the presence of incomplete Freund's adjuvant. Thus, we will couple the peptides to various carriers in an effort to improve immunogenicity. The immunological properties of the synthetic peptides are currently being assessed for protective and diagnostic utility.

SUMMARY

The antigenic structure of *Coxiella burnetii* is being investigated by identifying both external and internal cellular epitopes of the morphologic cell types. Both the phase I lipopolysaccharide (LPSI) and several surface proteins are candidates for the development of subunit multivalent vaccines. The protective efficacy of purified LPSI was demonstrated in A/J mice. The purified LPSI preparations contained residual peptides detected by amino acid analysis. Therefore, the protection afforded by LPSI may be, in part, due to the presence of peptides. The purification of proteins free of LPSI must be accomplished before the protective efficacy of proteins or peptides can be established. We have identified three proteins that are both antigenic and immunogenic, as indicated by either enzyme immunoassay, radioimmunoprecipitation, immunoblot assay, or lymphocyte transformation.

A 62-kDa protein antigen encoded by the *htpB* gene of *C. burnetii* was analyzed for immunogenicity. The purified protein antigen was immunogenic, as it

elicited specific antibodies and performed as recall antigen in lymphocyte stimulation assays. The antigen was not detected on the surface of phase I cells but was highly represented on the surface of phase II cells. Therefore, the protein may not be a good candidate for vaccine development. The diagnostic utility of the 62-kDa protein antigen lies in the fact that convalescent and chronic Q fever sera from human patients reacted with the antigen, whereas acute sera did not. Although the 62-kDa protein is a "common antigen," specific peptide-based diagnostic reagents may be useful in the detection of Q fever disease progression.

A major surface protein (P1) of roughly 29.5 kDa was purified from the phase I Nine Mile (clone 7) strain. No LPSI was detected in the P1 preparation by three different LPSI monoclonal antibodies. Monoclonal antibodies prepared against P1 were effective in localizing the protein on the cell surface, in the cell wall, and associated with the peptidoglycan of large cells of *C. burnetii*. Small, pressure-resistant cells did not contain P1. Mice immunized with two 25- μ g injections of LPSI produced antibodies against LPSI and phase I whole cells. No antibody was detected against phase II whole cells. Immunization with P1 induced antibody against the LPSI fraction and phase I and phase II whole cells. P1 was more effective than LPSI in reducing the number of infectious *C. burnetii* in the spleens of challenged mice.

The gene encoding another protein (P2) recognized by P1 monoclonal antibodies was cloned and sequenced. Although the P1 monoclonal antibodies recognized the recombinant *Escherichia coli* colony expressing P2, the two proteins were different. The nucleotide sequence of the gene for P2 revealed an unprocessed lipoprotein of 28.9 kDa or a mature lipoprotein of 27.1 kDa. The amino acid composition of P1 is significantly different than the predicted composition of P2. Beginning with the amino terminus, five synthetic peptides were prepared from the predicted amino acid sequence of P2. Peptides A, B, and C were recognized by immune mouse sera. The amino-terminal peptide, peptide A, stimulated an antibody response which recognized the surface of phase II whole cells. Peptides A, B, C, and D stimulated an anti-peptide response. The recognition of three of these peptides by immune mouse sera indicated that P2 was an immunogenic *C. burnetii* protein. Experiments in progress should determine the vaccinogenic potential of P2 and its peptides as synthetic vaccines and diagnostic reagents which will be free of LPSI.

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