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Humoral Immune Response to Q Fever: Enzyme-Linked Immunosorbent Assay Antibody Response to *Coxiella burnetii* in Experimentally Infected Guinea Pigs

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The response of guinea pigs experimentally infected with *Coxiella burnetii* organisms, the etiologic agents of Q fever, was obtained by the measurement of fever, circulating infectious *C. burnetii* cells, and anti-*C. burnetii* antibodies. The detection of antibodies by the enzyme-linked immunosorbent assay (ELISA) and traditional methods against phase I whole cells, phase II whole cells, and phase I lipopolysaccharide (LPS-I) (a virulence marker for phase I cells) antigens in the serum samples of infected animals revealed marked differences between intrastrain phase variants. Animals infected with the phase I Nine Mile strain produced a concomitant increase in temperature, circulating infectious *C. burnetii* cells, and antibodies against phase II cells, phase I cells, and LPS-I. At 15 weeks, a challenge of phase I-infected animals with viable phase I cells resulted in anamnestic antibody responses to phase I cells and LPS-I but not to phase II cells. Infection of animals with the phase II Nine Mile strain produced antibodies against only phase II cells. The challenge of phase II-infected animals at 15 weeks with viable phase II cells resulted in anamnestic antibody responses to phase I and phase II cells but not to LPS-I. Suppression of anti-phase II responses by the phase I challenge was apparent with only the ELISA, because the immunofluorescence, microagglutination, and complement fixation assays were insensitive to these changes. The sensitivity and specificity of the ELISA with whole-cell and the LPS-I antigens in the detection of phase-specific antibody revealed that avirulent phase II cells induced an immune response to phase I antigenic epitopes. Although the avirulent phase II cells were rapidly cleared by the host immune responses, they were sufficiently infective to induce antibody responses to both phase variants. Thus, in the occurrence of Q fever, any conventional serological technique that uses only phase II antigens may not provide a true incidence of naturally acquired infection with both phase I and II *C. burnetii* organisms.

The infection of laboratory animals with virulent phase I cells of *Coxiella burnetii*, the etiologic agent of Q fever, leads to progressive but usually self-limiting disease which is confirmed by the serological measurement of anti-*C. burnetii* antibodies (11, 16). While the fever course of phase I infection is detected early (3 to 7 days), no fever response occurs after infection with phase II *C. burnetii* organisms. Also, the infection or vaccination of experimental laboratory animals with phase I *C. burnetii* cells induces early (3 to 12 days) development of antibodies against avirulent phase II whole cells, whereas antibodies to phase I whole cells are produced late (12 to 30 days) (16, 19). The early detection of anti-*C. burnetii* antibodies against whole-cell phase II antigen is generally measured by the complement fixation assay (CFA) (19), microagglutination assay (MAA) (11), and indirect immunofluorescence assay (IFA) (16) with trichloroacetic acid- or *meta*-periodate-treated phase I whole cells, which convert phase I antigens to phase II antigens (4, 11). The sensitivity of specific antigenic determinants to each of these chemical treatments has not been evaluated. Therefore, the use of native Formalin-inactivated phase I and phase II whole cells as antigen for the serological detection of anti-*C. burnetii* antibodies is preferable to the use of chemically treated antigens.

In our companion study (23), we used an enzyme-linked immunosorbent assay (ELISA) to identify various antigenic fractions of phase I and phase II *C. burnetii* cells for the detection of phase-specific antibodies. With the use of immune sera, we determined that phase I and phase II whole cells were effective antigens, but a distinction between phase I and phase II antisera was possible only with antigenic fractions prepared with phenol water, ether, and *meta*-periodate extractions. The phase II antigens gave phase I specificity, whereas a correlation between antibody to phase II whole cells and a pure phase II soluble antigen was not possible with the *C. burnetii* fractions tested. Therefore, the objective of the current study was to evaluate the humoral immune response of guinea pigs after the injection of viable phase I or phase II *C. burnetii* cells with native phase I and phase II whole-cell antigens and the phase I lipopolysaccharide (LPS-I), a virulence marker of phase I cells. The development of fever, circulating infectious *C. burnetii* cells, and the humoral immune response of infected and homologously challenged guinea pigs was evaluated. The sensitivity and specificity of the native antigens in the detection of phase-specific antibodies by ELISA were compared with the results of conventional serological tests.

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TABLE 1. Detection by ELISA, IFA, MAA, and CFA of antibody against *C. burnetii* antigens after infection of guinea pigs with CB9MIC7 and C9MIIC4 organisms

Organism phase and test[a]	Antigen	Reciprocal titer after infection with phase I or phase II antigens on day[b]:										
		0	4[c]	6[c]	8[c]	12	16	20	28	40	55	68
I												
ELISA	Phase I	<10	<10	<10	10	320	640	1,280	2,560	2,560	2,560	5,120
	Phase II	<10	<10	10	160	10,240	40,960	81,920	20,480	20,480	10,240	5,120
	LPS-I	<10	<10	<10	<10	10	40	160	80	320	1,280	5,120
IFA	Phase I	<4	<4	<4	<4	<4	16	64	256	256	256	1,024
	Phase II	<4	<4	<4	<4	<4	2,048	16,384	16,384	4,096	1,024	512
MAA	Phase I	<4	<4	<4	<4	<4	4	8	64	32	16	32
	Phase II	<4	<4	<4	32	256	512	512	256	256	256	1,024
CFA	Phase I	<4	<4	<4	<4	<4	<4	8	64	64	64	128
	Phase II	<4	<4	<4	<4	16	512	1,024	512	1,024	256	128
	LPS-I	<4	<4	<4	<4	<4	<4	<4	<4	8	ND[d]	ND
II												
ELISA	Phase I	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
	Phase II	<10	<10	20	80	640	1,280	640	1,280	1,208	320	320
	LPS-I	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
IFA	Phase I	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4
	Phase II	<4	<4	<4	16	32	128	512	128	128	128	128
MAA	Phase I	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4
	Phase II	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4
CFA	Phase I	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4
	Phase II	<4	<4	<4	<4	<4	32	32	64	64	16	32
	LPS-I	<4	<4	<4	<4	<4	<4	<4	<4	ND	ND	ND

[a] Guinea pigs were prebled and then infected with 3.78×10^5 viable phase I or phase II *C. burnetii* organisms.
[b] Temperature and antibody responses were measured for 68 days after infection.
[c] Indicates a fever of >39.5°C. Rickettsemia was detected during the same time with only the phase I infections.
[d] ND, Not done.

MATERIALS AND METHODS

Preparation of antigens. Particulate and soluble antigens of phase I and phase II *C. burnetii* cells were derived from viable and Formalin-inactivated microorganisms purified from infected yolk sacs of embryonated chicken eggs (22). Phase-specific strains were the virulent Nine Mile phase I (CB9MIC7) (5, 8) and avirulent Nine Mile phase II (CB9MIIC4) (22).

Viable *C. burnetii* whole cells were purified (22), inactivated with 1% Formalin, dialyzed against water, and lyophilized. Whole cells were chemically extracted and subfractionated by hot phenol water to obtain LPS-I (1).

ELISA. The technique for the ELISA was adapted from Engvall and Perlmann (9) as described in the companion paper (23).

Infection of guinea pigs with phase I and phase II viable *C. burnetii* cells. Two groups of six guinea pigs (Hartley strain; National Institute of Allergy and Infectious Diseases Rocky Mountain Laboratories, Hamilton, Mont.; 750 to 800 g, each animal) approximately 90 days of age were infected intraperitoneally with 1 ml of 3.78×10^5 purified viable phase I or phase II *C. burnetii* cells (22). Temperatures of the guinea pigs were recorded daily for at least 2 weeks after the initial injections and then on the day of each bleeding, except for the final bleeding. Blood specimens were collected on various days up to 105. On day 105, the animals in the phase I group were challenged with 10^5 live CB9MIC7 cells and the animals in the phase II group were challenged with 10^{10} live CB9MIIC4 organisms. Animals of both groups were bled on day 0 just before the challenge and on days 7, 37, and 76 after the challenge. These dates actually coincided with days 105, 112, 142, and 181 after the initial injection. Sera from the animals were filtered (0.22-μm pore) and sto· · at −20°C until tested for antibody.

Detection of *C. burnetii* cells in blood. The presence of *C. burnetii* cells in the blood specimens of infected animals was tested. Whole-blood samples obtained from each of the guinea pigs infected with phase I or phase II cells were collected before and after infection up to day 33. The specimens were triturated in 5 ml of brain heart infusion broth, and 0.5 ml of the suspension was injected intraperitoneally into each of four C57BL/10 ScN mice. Mice receiving an injection prepared from the guinea pig blood were bled after 22 or 23 days, and the blood samples were pooled. The mouse sera were filtered and stored at −20°C until tested for antibody.

RESULTS

Detection of fever and virulent *C. burnetii* organisms in the blood samples of infected guinea pigs. Animals were experimentally infected with phase I or phase II *C. burnetii* cells (Table 1). Average temperatures (>39.5°C) were obtained on days 4, 6, and 8 after the intraperitoneal injection of guinea pigs with phase I *C. burnetii* organisms. An increase in temperature was not observed after the phase II cell injection of guinea pigs. Evidence for phase I *C. burnetii* cells in

the blood samples of guinea pigs was obtained by injecting mice with whole blood. Whole blood samples from infected guinea pigs collected on days 4, 6, and 8 contained viable *C. burnetii* organisms, as evidenced by the seroconversion of mice injected with these samples (Table 1). There was no serological evidence from mice of phase II *C. burnetii* cells in the blood specimens of guinea pigs inoculated with live phase II whole cells. Significant ELISA titers were detected in sera from all mice exhibiting splenomegaly after the injection of phase I *C. burnetii* organisms (data not shown). The avirulent phase II strain could not by standard techniques (8) be isolated from the spleens or blood samples of injected guinea pigs or mice.

Comparison of ELISA, IFA, MAA, and CFA. The sensitivity and specificity of four serological tests for antibodies against phase I and phase II *C. burnetii* whole cells and LPS-I were compared by using the pooled sera from six guinea pigs per group. Antibodies against phase II whole cells were obtained with the ELISA by day 6 and with the other three serological tests by day 8 after infection with phase I cells. Antibodies against phase I whole cells were detected by day 8 by the ELISA, day 16 by the IFA and MAA, and day 20 by the CFA. Antibodies against LPS-I were detected by day 12 by the ELISA and by day 40 by the CFA.

The infection of guinea pigs with phase II *C. burnetii* cells induced detectable antibody responses to only phase II whole cells by day 6 by the ELISA, day 8 by the IFA, and day 16 by the CFA; no antibodies were detected by the MAA. After animal infection with phase II cells, the ELISA indicated that the anti-phase II titers were not as high as with phase I infection, but they persisted to 68 days.

Anamnestic responses after homologous challenge. After the phase I challenge of guinea pigs previously infected with phase I organisms (Table 2), significant increases in antibody titers against phase I and phase II cells and LPS-I were observed during two of the four serological tests. The ELISA was the most sensitive test that detected significantly greater titers against the phase I and LPS-I antigens. Suppression or lack of enhancement of anti-phase II titers was more easily detected with the ELISA.

Antibody responses to the phase II antigen were specific for phase II organisms in guinea pigs previously injected with phase II cells and subsequently challenged with the same. The IFA, MAA, and CFA showed specific increases in anti-phase II titers. The ELISA was the only test that detected a marked increase in anti-phase I and -phase II antibody titers with both the phase I and phase II antigens without an increased titer against the LPS-I antigen.

TABLE 2. Comparison of anti-*C. burnetii* titers after challenge

Organism phase and test[a]	Antigen	Reciprocal titers on day[b]:			
		0	7	37	76
I					
ELISA	Phase I	10,240	20,480	710,720	40,960
	Phase II	1,280	2,560	10,240	2,560
	LPS-I	20,480	91,920	327,680	40,960
IFA	Phase I	64	640	10,240	1,024
	Phase II	64	640	320	256
MAA	Phase I	8	32	128	64
	Phase II	8	8	32	16
CFA	Phase I	128	64	1,280	2,048
	Phase II	64	32	128	64
	LPS-I	4	8	32	64
II					
ELISA	Phase I	<10	32	20,480	5,120
	Phase II	320	80	81,920	81,920
	LPS-I	<10	<10	<10	<10
IFA	Phase I	<4	<4	<4	<4
	Phase II	32	64	51	1,024
MAA	Phase I	<4	<4	<4	<4
	Phase II	<4	<4	64	256
CFA	Phase I	<4	<4	<4	<4
	Phase II	<4	<4	256	2,048
	LPS-I	<4	<4	<4	<4

[a] Guinea pigs (see Table 1) were homologously challenged with viable phase I and viable phase II cells on day 105 after the initial infection.

[b] Sera were collected just before the challenge on day 0 (day 105 postinfection) and after the challenge on the other days indicated.

DISCUSSION

To evaluate two *C. burnetii* Nine Mile strains for their ability to infect guinea pigs, we studied the temporal development of fever, circulating infectious *C. burnetii* cells, and antibody. The virulent phase I strain (CB9MIC7) produced a fever response and infectious *C. burnetii* organisms from days 4 to 8 after injection. The avirulent phase II strain (CB9MIIC4) did not produce a fever response or detectable infectious organisms in the blood of injected guinea pigs. Attempts to isolate the avirulent strain from the spleens of infected guinea pigs were also unsuccessful.

The development of specific antibody after phase I or phase II infection indicated that the ELISA detected antibodies earlier in the infection and gave greater endpoint titrations than did other conventional methods. After phase I infection, the detection of an early antibody response to the LPS-I antigen, a virulence marker of phase I cells, was possible only with the ELISA. The difference in titer measured by phase I whole cells and LPS-I indicated that other non-LPS-I determinants of the phase I whole cells were measured by the ELISA. Although other sensitive serological tests have been developed (2, 20), they are not used routinely. In concordance with other serological tests (3, 11, 13, 14, 15, 18, 19, 21), the first antibodies detected in the ELISA were against phase II whole cells.

The injection of animals with viable, avirulent phase II cells produced detectable antibody responses only against phase II cells when the ELISA, IFA, and CFA were used. The ELISA detected antibodies against phase II cells by day 6 postinjection with phase I or phase II cells. After the injection of viable phase I or phase II *C. burnetii* organisms, the ELISA antibody titers against phase II cells reached a peak on days 20 or 16, respectively. The antibody titers could not reasonably be thought to be the result of nonreplicating phase II *C. burnetii* cells, because the amount of antigen injected was only 10 ng, which is below the threshold of immunogenicity. The phase I cells were virulent and produced high antibody titers, but the extent and duration of the phase II antibody response after the injection of viable avirulent phase II antigens was unexpected. This may be the result of an initial replicative burst of phase II *C. burnetii* organisms, followed by a brisk clearance of these avirulent cells. These results suggest that phase II *C. burnetii* cells are successful parasites in immunocompetent animals. One might expect to find naturally occurring, as

opposed to laboratory derived, phase II *C. burnetii* organisms that can initiate a mild infection. Also our results raised the possibility that *C. burnetii* organisms traditionally considered as phase II cells may cause both acute and chronic infections. The isolation of virulent phase I microorganisms by standard laboratory techniques (16) is clearly biased against the isolation of naturally occurring phase II *C. burnetii* organisms, because immunocompetent animals clear most phase II strains. The generally held view that phase II avirulent cells are just a laboratory curiosity should be modified until either naturally occurring or other cloned intrastrain phase I and phase II variants are studied.

Anamnestic antibody responses to phase-specific antigens were measured after homologous challenge with viable phase I or phase II cells. ELISA antibody titers against LPS-I and phase I whole cells were markedly increased in animals previously infected with phase I cells. However, antibody titers to phase II cells were only slightly increased and never reached the phase I titers. The suppression of an anti-phase II response in the animals previously infected with phase I cells and then challenged with phase I cells was unexpected. This is the only experimental example of the suppression or lack of enhancement of specific antibody responses against phase II antigens after the secondary infection of animals with phase I *C. burnetii* organisms. The nature of this apparent suppression of antibody responses requires further study.

The results of the IFA, MAA, and CFA did not correlate with those of the ELISA, suggesting that the more sensitive ELISA detected different epitopes from those found by other assays. This assumption was further substantiated by the fact that the phase II homologous challenge produced a marked increase in ELISA titers to the phase I and phase II cells, whereas the IFA, MAA, and CFA detected antibody increases for only the phase II cells. No antibody response was observed against LPS-I after the phase II homologous challenge. Therefore, the lack of suppression of antibodies to phase II cells in animals homologously challenged suggests that phase I infection induces the suppression of antibody responses to specific phase II antigens but enhances antibody responses to phase I-specific antigens. Virulent phase I cells previously were shown to induce immunosuppression of murine splenic lymphocytes, whereas phase II cells were not effective (7). The current study is the first to suggest that phase I cells can also modulate the synthesis of specific antibody.

The objective of an ELISA for Q-fever detection is to determine the anti-*C. burnetii* phase-specific antibody responses. The diagnostic utility of this antibody assay lies in the determination of the ratio of phase II to phase I reactivity (16) so that seroepidemiological studies can reveal the acute and chronic forms of the disease. The ratios of phase II to phase I cells at ELISA endpoint titration should be predictive of the disease state, as demonstrated previously with the MAA, CFA, and IFA (16). The value of an ELISA for Q-fever detection may be lost if only a single figure is given as a qualitative compositional analysis of phase-specific antibodies. By an analysis of ELISA dose-response curves, an estimate of the total specific antibodies of the subclasses is possible. The ELISA should be a more sensitive assay for the antigenic comparison of strains (12). Recently, others (6, 9, 10, 16, 17, 24) have used ELISA procedures for Q-fever detection with phase I or phase II whole cells prepared by various extraction procedures. In a companion study (23), we showed that certain antigens were extracted by the purification methods of other investigators. We recommend that phase I and phase II whole cells and LPS-I be used as standard antigens in the ELISA for Q-fever detection. In this way, results from different laboratories can be more readily compared.

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