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Characterization of a Phase I *Coxiella burnetii* Chloroform-Methanol Residue Vaccine That Induces Active Immunity against Q Fever in C57BL/10 ScN Mice

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The effect of phase I *Coxiella burnetii* chloroform-methanol residue vaccine (CMRV) on the response of murine splenic lymphocytes to mitogenic and antigenic stimuli was evaluated in C57BL/10 ScN endotoxin-nonresponder mice with an in vitro lymphocyte proliferation assay. Intraperitoneal injection of phase I CMRV resulted in antibody production against phases I and II antigens. Lymphocytes were responsive in vitro to concanavalin A, phytohemagglutinin, pokeweed mitogen, and specific recall antigens. Antibodies against phases I and II antigens were not detected after intraperitoneal injection of chloroform-methanol extract (CME). Lymphocytes also were only slightly hyporesponsive to mitogens. Reconstitution of the CMRV with the CME of phase I whole cells restored the immunopathological reactions that were associated with the phase I whole cell vaccine (WCV). The CMRV was more mitogenic than the WCV for lymphocytes from mice injected with saline. Lymphocytes from phase I WCV-injected mice were negatively modulated with nontoxic concentrations of homologous WCV or CMRV. Lymphocytes from phase I CMRV-injected mice were only slightly hyporesponsive to mitogens and were significantly stimulated by antigens of either WCV or CMRV as recall antigens. Vaccination of mice with 100 μg of CMRV, CME, or WCV provided 80, 0, or 50% protection, respectively, against a lethal intraperitoneal challenge with viable phase I *C. burnetii*. The epitopes which induce immunological hyporesponsiveness, negative modulation, and the death of lymphocytes were fractionated into the CMRV and CME. The CMRV provides at least one of the determinants which induce immunosuppression, whereas CME contains specific or nonspecific components or both. Collectively, these results show that the CMRV may be a potential candidate to replace the WCV currently used for human vaccination.

Infection of individuals with phase I *Coxiella burnetii*, the etiological agent of Q fever, results in the development of serum antibodies and of cell-mediated immunity, which are readily detectable by various in vivo and in vitro assays. Protection against natural or experimentally induced infection is obtained by vaccination with suspensions of killed phase I whole-cell vaccines (WCV) which induce immunopathological reactions in humans and animals (5, 27). Previous studies on the safety and efficacy of WCV have centered around a determination of the acceptable dosage of vaccine based on side effects, on antibody conversion rate (29), and on the detection of cell-mediated immunity with a delayed-type skin test (3, 20) or in vitro lymphocyte proliferation assay (14). Adverse reactions obtained during vaccine trials have prevented the widespread and unconditional use of phase I WCV. Attempts to remove components which induce adverse reactions have met with limited success because partially purified phase I antigens obtained by various organic or aqueous solvent extractions were effective immunogens only when used with adjuvants (for a review see reference 5). Thus, the relationship between phase I WCV and immunogenic fractions which induce deleterious tissue reactions, delayed-type hypersensitivity, in vitro lymphocyte proliferation, and resistance to infection is not known.

Recent studies have shown that immunization of C57 BL/10 ScN endotoxin-nonresponder mice with phase I WCV of *C. burnetii* induced immunopathological reactions (9, 32). Immunization with phase I WCV induced a time- and dosage-dependent lymphocyte unresponsiveness which correlated with the appearance of hepatomegaly and splenomegaly and with death of the mice (32). Concurrently, generalized nonspecific splenic and lymph node lymphocyte hyporesponsiveness was developed to concanavalin A (ConA), phytohemagglutinin-P (PHA), and pokeweed mitogens (PWM) (9). Splenic and lymph node lymphocytes from immunized mice were down regulated below normal control levels by specific recall antigen. In these studies cellular expression of negative modulation was a property of phase I *C. burnetii* since phase II cells were not effective in the induction of lymphocyte unresponsiveness (9). Components of the phase I WCV which were not required for protective efficacy were successfully extracted with chloroform-methanol (32). In the present study, we show that a chloroform-methanol residue vaccine (CMRV) prepared

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from phase I WCV did not induce either immunopathological reaction or suppression of mitogenic and antigenic lymphocyte proliferation after intraperitoneal injections of C57BL/10 ScN mice. The ability of CMRV to induce in vitro lymphocyte proliferative responsiveness correlated with the protective efficacy of the vaccine. Furthermore, deleterious tissue reactions and suppression of mitogenic and antigenic lymphocyte responsiveness occurred when CMRV was reconstituted with chloroform-methanol extract (CME). Thus, virulence factors required for the adverse reactions of phase I *C. burnetii* were fractionated into both the CME and the CMRV.

MATERIALS AND METHODS

***C. burnetii* antigens.** *C. burnetii* Ohio strain, phase I, was propagated, purified, and inactivated with formaldehyde as previously described (34). Extraction of lyophilized whole cells with chloroform-methanol (4:1) (32) produced a particulate material (chloroform-methanol residue [CMR]) and extracted components (CME).

Reconstitution of CMR with CME was performed by suspending CMR (3.6 mg) with CME (1 mg) in chloroform-methanol (4:1) at a ratio of approximately four parts of CMR and one part of CME. The suspension was treated in an ultrasonic cleaner (model 8845-30; Cole-Parmer) for 5 min and incubated at room temperature for 48 h. This suspension was dried under a stream of nitrogen, and the residue was suspended in saline or tissue culture medium with the aid of an ultrasonic cleaner. Another reconsitution was carried out with 1 mg of CME in chloroform-methanol (4:1), but the CME sample was dried under a stream of nitrogen before adding the CMR suspended in saline. Resuspension was carried out in an ultrasonic cleaner for 5 min.

Chemical analyses of the WCV, CMRV, and CME. Amino acids, total phosphorus (21), total neutral sugars (10), 3-deoxy-D-manno-octulosonic acid (KDO)-like components (25), and total fatty acids were analyzed as previously described (1).

Immunization. C57BL/10 ScN endotoxin-nonresponder (8, 23) mice (Harlan Sprague-Dawley, Inc., Indianapolis, Ind.) were injected intraperitoneally (i.p.) in groups of 3 to 10 mice with 0.5 ml of saline containing various concentrations of either WCV, CMRV, CME, or CMRV reconstituted with CME.

Immunological assays. Graded doses of vaccine preparations were evaluated for the induction of pathological responses and immunity as follows: the measurement of hepatosplenomegaly (32); the induction of immunosuppression based on the incorporation of [^{3}H]TdR (specific activity, 5 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) by splenic lymphocytes in response to mitogens and recall antigens; and the induction of immunity based on splenic lymphocyte responses to recall antigens (9), production of phases I and II antibodies (anti-phase I and anti-phase II) (11), and protection against a lethal i.p. challenge dose of phase I *C. burnetii* (32).

Responses of nucleated spleen cells under various experimental conditions were expressed as stimulation indices (SI) and percent suppression (% Supp) as follows: SI = (counts per minute in mitogen- or antigen-stimulated cells/counts per minute in unstimulated cells); % Supp = [1 − (SI of cells from treated mice/SI of cells from control mice)] × 100.

Immunofluorescence identification of *C. burnetii*. During necropsy, whole spleens were taken and cut with a razor blade, and three impressions were made on glass slides. Spleen impressions were air dried and fixed in acetone for 15 min. Fluorescein-tagged guinea pig anti-*C. burnetii* serum against both phases I and II were used to detect *C. burnetii* antigen in the spleens of mice from either infected or vaccinated animals (13, 34).

RESULTS

Chemical compositions of fractions. Fractionation of the WCV into CMR and CME resulted in the recovery of roughly 80% as CMR and 20% as CME (32). Analyses of total protein by amino acid composition of each fraction indicated that the CMR contained about 16% less protein than the WCV, and the CME contained only 0.4% protein (Table 1). Phosphate, neutral sugars, and fatty acids were present in each fraction. Phosphorous concentrations were highest in whole cells but were roughly equivalent in the CMR and CME fractions. Neutral sugar concentrations were greatest in CMR and least in CME. Concentrations of KDO-like compounds were greatest in the WCV and least in CME. The major portion of the fatty acids were fractionated into the CME. But the percentage of the total fatty acids represented by the branched chain fatty acids in each fraction was very similar.

Relative effectiveness of WCV, CMRV, and CME in eliciting splenomegaly, specific antibody, and lymphocyte responsiveness. The effects of a single i.p. injection of saline or of 10, 100, and 1,000 μg of either WCV or CMRV on spleen weight, specific antibody response, and mitogenic activity were evaluated in C57BL/10 ScN mice. Animals treated with WCV showed a significant spleen weight increase which was dependent on time and dosage (Fig. 1A). Increase in spleen weight was detected by day 3 postinjection and reached a maximum by day 21 for the 10- and 1,000-μg dosage and days 14 to 21 for the 100-μg dosage. Transient splenomegaly was noted for the 10- and 100-μg dosage, whereas the 1,000-μg dosage produced splenomegaly throughout the 35-day experimental period. Animals injected with CMRV (Fig. 1B) and with 100 μg of CME (data not shown) showed no splenomegaly.

The temporal sequences of anti-phase I and anti-phase II antibodies after the i.p. injections of saline or of 10, 100, and 1,000 μg of WCV and CMRV were compared (Table 2). The anti-phase II response was detected on day 3 postinjection at all three dosages and continued to increase until 35 days postinjection. Anti-phase I activity was detected by days 7 and 14 postinjection of either 100 or 1,000 μg of WCV and CMRV, and specific anti-phase I activity continued to in-

TABLE 1. Chemical analyses of phase I *C. burnetii* whole cells, CMR, and CME

Fraction	Component measured					
	Total protein (mg/100 ml [dry wt])	Total phosphorus (nmol/mg)	Neutral sugar (nmol/mg)	KDO-like compound[a] (nmol/mg)	Total fatty acids (% of total)	Branched chain fatty acids (% of total)
WCV	53	738	527	26	14	70
CMRV	45	523	699	18	3	76
CME	0.4	453	392	6	12	68

[a] Thiobarbituric acid positive (25). For an explanation of KDO-like compound see reference 25.

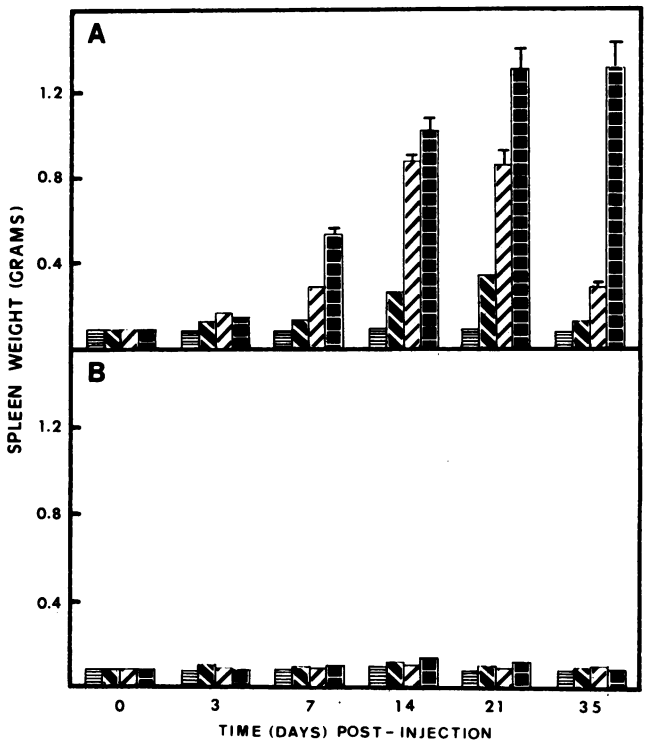

FIG. 1. Biological activity of *C. burnetii* WCV or CMRV in C57BL/10 ScN mice. Effect on splenomegaly of saline, 10, 100, or 1,000 μg of WCV or CMRV with respect to time after a single i.p. injection of the vaccines. (A) WCV and (B) CMRV; ▤, saline; ▧, 10 μg; ▨, 100 μg; and ▥, 1,000 μg.

crease until 35 days postinjection. On day 14 postinjection of 10 μg of WCV, anti-phase I activity was detected, and this titer continued to increase until 35 days postinjection. In sharp contrast was the lack of anti-phase I activity up to 35 days postinjection with the 10-μg dosage of CMRV. These comparisons between vaccine dosage and anti-phase I and anti-phase II activities suggest that adjuvant-active components extracted from the WCV by chloroform-methanol may facilitate polyclonal antibody responses.

Elicitation of anti-phase I and anti-phase II activities with WCV and CMRV was related to the persistence of antigen in the spleens of injected mice. Clearance of WCV antigen by mice was less effective since antigen was detected in spleen impression smears 35 days postinjection, whereas CMRV antigen was not detected (data not shown).

Responses of splenic lymphocytes from vaccinated mice to the mitogens ConA, PHA, and PWM were tested in microplate cultures. Mice receiving 100 μg of CMRV showed a transient suppression of the mitogenic activity of ConA and PHA on days 3 and 7, but the mitogenic activity returned to normal values by day 14 (Fig. 2, ConA and PHA). Suppression of the mitogenic activity of PWM was not detected (Fig. 2, PWM). The transient suppression observed with this CMRV preparation was attributed to inadequate chloroform-methanol extraction of the WCV. Extraction of WCV five times with chloroform-methanol resulted in a CMRV which did not induce marked suppression on day 4 postinjection (Table 3).

Mitogen-induced lymphocyte proliferation of normal, untreated spleen cell suspensions from saline-injected mice gave SI for ConA, PHA, and PWM of 43, 9, and 19, respectively (Table 4). Lymphocytes from mice receiving 100 μg of WCV gave SI for ConA, PHA, and PWM of 8, 2, and 6, respectively. Lymphocytes from mice receiving 100 μg of CMRV gave SI for ConA, PHA, and PWM of 45, 9, and 17, respectively. Thus, injection of mice with CMRV did not suppress the proliferation response of lymphocytes to mitogens; whereas WCV produced a persistent suppression of lymphocyte activity to ConA, PHA, and PWM by 81, 78, and 68%, respectively. This persistent suppression of lymphocyte activity elicited by WCV seemed to correlate with the capacity of WCV to induce splenomegaly (Fig. 1A). Injection of CMRV, on the other hand, resulted in enhancement of lymphocyte activity to ConA (−5%), a slight suppression of lymphocyte activity to PWM (11%), and no effect on lymphocyte activity to PHA.

Effect of WCV and CMRV on lymphocyte responses. Mitogenic activity of WCV and CMRV was tested with normal

TABLE 2. Serological response in C57BL/10 ScN mice immunized with *C. burnetii* WCV, CMRV, or CME

Treatment and dosage (μg)	Microagglutination titer at days postinjection[a]									
	3		7		14		21		35	
	I[b]	II[c]	I	II	I	II	I	II	I	II
Saline	0	0	0	0	0	0	0	0	0	0
WCV										
10	0	4	0	5	1	7	4	7	5	9
100	0	7	1	7	2	8	6	9	6	9
1,000	0	8	2	10	4	10	5[d]	12	5.5[d]	12
CMRV										
10	0	4	0	5	0	7	0	6	0	2
100	0	5	1	7	2	8	3	7	2	7
1,000	0	7	1	8	2	9	4	8	2	7
CME										
100	0	0	0	0	0	0	0	0	0	0

[a] MA titer expressed as $\log_2$, with the error of detection in this test of ±1 log unit. Results are expressed as geometric means of three to five serum samples.
[b] I, Phase I WCV antigen.
[c] II, Phase II WCV antigen.
[d] From one surviving mouse at each period.

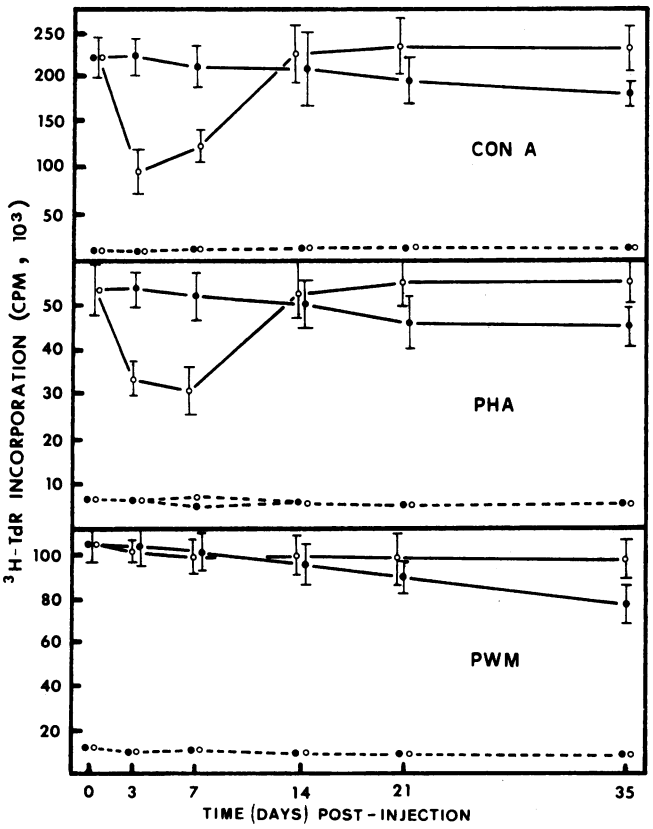

FIG. 2. Splenic lymphocyte responses to mitogens after a single i.p. injection of C57BL/10 ScN with saline or 100 μg of CMRV. ConA, PHA, and PWM were used at 1, 10, and 10 μg/ml, respectively. Data points represent the arithmetic mean of quadruplicate counts conducted on a pooled suspension of spleen cells from three mice; ○, 100 μg of WCV; ●, saline; bars, range.

splenic lymphocyte suspensions (Fig. 3). A dose-response curve of WCV or CMRV indicated that maximal mitogenic activity was obtained with a dosage of between 50 and 100 μg of WCV per ml, and a plateau was achieved at a dosage of 100 μg of CMRV per ml. Thus, CMRV was roughly threefold more mitogenic than the WCV based on weight and chemical composition (Table 1). These data suggest that either WCV was less mitogenic than CMRV or that WCV contained components that decreased the activity or viability of mouse splenic lymphocytes.

Lymphocytes from normal and immunized mice were tested in an antigenic lymphocyte proliferation assay (Table 5). Mice were immunized with 100 μg of WCV or CMRV, and in vitro cultures of splenic lymphocytes were mixed with 0, 5, 50, and 500 μg of the respective antigens per ml. The WCV and CMRV at 5 and 50 μg/ml, respectively, were mitogenic for normal splenic lymphocytes (Table 5). Negative modulation of splenic lymphocyte responses to antigen was obtained with WCV-injected mice, whereas antigen-induced lymphocyte proliferation was obtained with CMRV-injected mice (Table 5). Cultivation of lymphocytes from normal, WCV-, and CMRV-injected mice with 500 μg of either WCV or CMRV per ml indicated that the cells were either unresponsive or were negatively modulated by the antigens. Corrections of counts per minute (cpm) per well to cpm per viable cell per well indicated that a SI <1.0 correlated with a reduction in the number of viable cells for the treatment with 500 μg per well. However, lymphocytes from WCV-injected mice which gave a SI <1.0 in the presence of 5 and 50 μg of WCV or CMRV per ml showed no significant reduction in viable cells (Table 5). This demonstrates that a decrease in the cpm per well was not a direct response to cytotoxic activities during the in vitro cultivation of cells with 5 μg of either WCV or CMRV recall antigens per ml.

Reconstitution of CMRV with CME. Since both CMRV and CME injected separately did not induce splenomegaly or suppression of lymphocyte activity, we tested the hypothesis that reconstitution of CMRV with CME would induce both immunopathological responses and suppression of lymphocyte activity. Reconstitution of CMRV with CME in phosphate-buffered saline did not induce marked splenomegaly or suppression of lymphocyte activity (data not shown), whereas reconstitution in chloroform-methanol induced marked splenomegaly and suppression of lymphocyte activity (Table 6). Injection of CMRV produced enhanced mitogenic activity of lymphocytes without splenomegaly. Injection of CME alone induced neither splenomegaly nor antigenic negative modulation of splenic lymphocytes (data not shown), but it did induce slight mitogenic lymphocyte hyporesponsiveness (Table 6).

Protection against a lethal challenge dose of viable phase I *C. burnetii* cells. The protective efficacy of a dosage of 100 μg of either WCV, CMRV, or CME was tested in mice (Table 7). Immunized mice were challenged i.p. 14 days postinjection with phase I *C. burnetii* at a dosage of 7×10^{10} PFU per mouse (32, 34). WCV protected 50% of the mice, CMRV protected 80% of the mice, and CME was not efficacious. Multiple injections with CMRV were therapeutic since they gave partial protection against the immunopathological changes associated with prior or subsequent injections of WCV or against challenge by viable phase I *C. burnetii* cells (D. M. Waag and J. C. Williams, unpublished data).

TABLE 3. Effect of extensive chloroform-methanol extraction of CMRV on the suppression of mitogenic responsiveness of C57BL/10 ScN spleen cells

In vivo antigen[a] and in vitro mitogen[b]	Response of spleen cells after treatment with:				% Supp[d]
	Saline (0.5 ml)		CMRV (100 μg)		
	cpm	SI[c]	cpm	SI	
CMRV, 3×					
None	5,227 ± 261		3,981 ± 199		
ConA	236,791 ± 9,471	45	106,418 ± 4,256	27	40
PHA	59,047 ± 2,952	11	30,027 ± 900	8	27
PWM	101,349 ± 4,503	19	73,937 ± 3,696	19	0
CMRV, 5×					
None	4,594 ± 183		4,151 ± 207		
ConA	215,856 ± 6,475	47	175,795 ± 7,031	42	11
PHA	53,233 ± 2,129	12	46,027 ± 2,301	11	8
PWM	96,252 ± 3,850	21	86,917 ± 2,607	21	0

[a] Three mice in each group were injected with either 100 μg of CMRV prepared by three reflux treatments of WCV with chloroform-methanol or with an equivalent amount of CMRV extracted five times.

[b] On day 4 postinjection of CMRV, spleen cells from injected mice were collected and stimulated in vitro with an optimal mitogenic dose of ConA, PHA, or PWM. Data are expressed as the mean cpm plus or minus the standard error of incorporation of [^{3}H]TdR in quadruplicate cultures.

[c] SI (cpm in mitogen-stimulated cultures/cpm in unstimulated cultures).

[d] % Supp = 1 − (SI of cells from treated mice/SI of cells from control mice) × 100.

TABLE 4. Mitogenic responses of C57BL/10 ScN spleen cells to ConA, PHA, and PWM after a single injection of saline, WCV, or CMRV

Mouse treatment[a]	Response as incorporation of [^{3}H]TdR								
	Nonstimulated cultures (cpm)[b]	Cultures stimulated with:							
		ConA			PHA			PWM		
		cpm	SI[c]	% Supp[c]	cpm	SI	% Supp	cpm	SI	% Supp
Saline	5,651 ± 364	242,402 ± 34,689	43		51,106 ± 9,892	9		106,267 ± 13,205	19	
WCV	4,236 ± 281	35,904 ± 4,349	8	81	6,942 ± 782	2	78	26,038 ± 3,011	6	68
CMRV	6,031 ± 279	268,434 ± 38,413	45	−5	54,118 ± 9,624	9	0	102,860 ± 12,781	17	11

[a] Three mice in each group were given a single 0.5-ml injection i.p. of saline containing 100 μg of WCV or CMRV. Animals were assayed for mitogenic responsiveness 2 weeks later.
[b] Mean cpm plus or minus the standard error of incorporation of [^{3}H]TdR in quadruplicate cultures.
[c] For an explanation of SI and % Supp, see Table 2, footnotes *d* and *e*.

DISCUSSION

Phase I *C. burnetii* WCVs are potent modulators of cellular mediated immune responses that result in immunity to infection and in immunopathological reactions. Although the protective efficacy of the WCVs has been demonstrated, the adverse properties of the phase I WCVs have been verified in humans (3, 20, 29), guinea pigs (2), and mice (9, 32). The chemical composition of the subfractions of *C. burnetii* responsible for these immunopathological properties has not been characterized. Our interest in subfractions of *C. burnetii* that elicit protective immunity without inducing deleterious tissue reactions follows from a previous study which showed that CMRV was efficacious in mice (32). In the present study, the immunomodulatory activities of WCV, CMRV, and CME were compared. The results indicated that i.p. injection of mice with CMRV did not induce suppression of mitogenic or antigenic lymphocyte proliferation. The suppressive activity of phase I WCV in C57BL/10 ScN mice (9) results in mitogenic hyporesponsiveness and antigenic-negative modulation of splenic lymphocytes, affecting the in vitro replication of both B and T cells. The negative regulatory activity of phase I *C. burnetii* on previously sensitized splenic lymphocytes suggests that WCV was able to directly regulate lymphocyte activity. Evaluation of the immunogenic potential of CMRV indicated that the immunosuppressive and pathologic activity was successfully extracted by chloroform-methanol (Fig. 2, Tables 3 and 4), but these activities were reconstituted by recombining CMRV with CME (Table 6). In addition, CMRV was more mitogenic for normal splenic lymphocytes than WCV was on a weight basis (Fig. 3). It is noteworthy that the extraction of adjuvant-active materials by chloroform-methanol did not reduce the prophylactic efficacy of the CMRV, which was equal to or slightly greater than that of the WCV (Table 7).

The suppressive activity exhibited by phase I *C. burnetii* vaccine may be a useful correlate of pathologic behavior during *C. burnetii* infection. Recent studies have shown that murine lymphocytes from infected animals are suppressed (9). Moreover, studies with peripheral blood lymphocytes from human Q fever patients with clinical signs of primary, hepatic, and endocarditic involvement suggest that suppressor cell activity either plays a role in the development or is a result of chronic Q fever (17). In those studies, the induction of suppression was antigen specific, but the suppressor mechanism was antigen nonspecific (17–19). Thus, antigen-specific lymphocyte unresponsiveness was apparently in

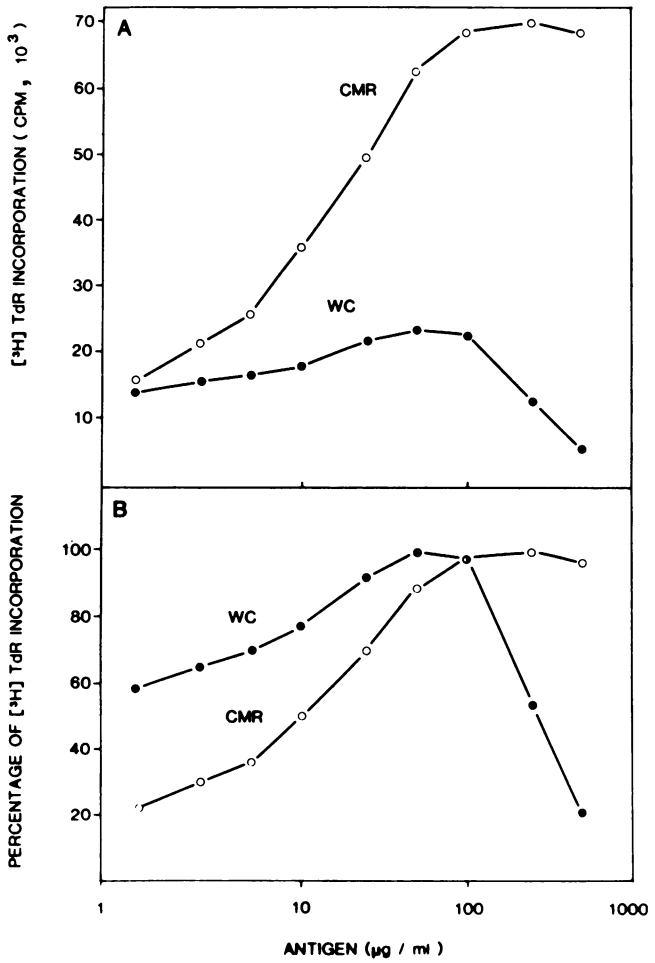

FIG. 3. Splenic lymphocyte responses to mitogens during in vitro incubation of C57BL/10 ScN normal spleen cells with *C. burnetii* WCV (WC) or CMRV (CMR). Spleen cells were prepared from uninjected mice and then incubated with varying concentrations of WCV or CMRV. Data points represent the arithmetic means of quadruplicate counts conducted on a pooled suspension from spleen cells for three mice. (A) Data expressed as [^{3}H]TdR incorporation. (B) Data in panel A recalculated as percentage of maximum activity.

TABLE 5. Effect of phase I *C. burnetii* WCV or CMRV on mitogenic and antigenic lymphocyte proliferative responses of spleen cells from C57BL/10 ScN mice injected with saline, WCV, or CMRV

Recall antigen (μg/ml)	Response as incorporation of [^{3}H]TdR with mouse treatment[a]								
	Saline			WCV			CMRV		
	cpm[b]	cpm/VC[c]	SI[d]	cpm	cpm/VC	SI	cpm	cpm/VC	SI
None (0)	4,661 ± 461	5,826		3,000 ± 322	5,000		3,583 ± 632	9,633	
WCV									
5	8,574 ± 1,198	8,574	1.8	296 ± 154	493	0.1	10,351 ± 161	8,626	2.7
50	13,979 ± 2,512	13,979	3.0	214 ± 68	357	0.1	14,969 ± 3,691	12,474	3.9
500	2,027 ± 209	5,068	0.4	161 ± 24	403	0.1	345 ± 144	863	0.1
CMRV									
5	11,712 ± 2,743	14,640	2.5	217 ± 78	362	0.1	15,135 ± 2,963	7,568	3.9
50	10,479 ± 1,003	10,479	2.2	184 ± 29	460	0.1	20,202 ± 2,469	10,101	5.2
500	2,175 ± 1,388	10,875	0.5	136 ± 23	680	0.05	1,767 ± 655	8,835	0.5

[a] Three mice in each group were injected i.p. with 0.5 ml of saline or with 100 μg of WCV or CMRV 2 weeks before assay.
[b] Data are expressed as the mean cpm plus or minus the standard error of [^{3}H]TdR incorporation in quadruplicate cultures from one experimental trial.
[c] cpm/VC (viable cell) ($\times 10^{-5}$) = cpm/well ÷ VC/well. VC/well = (total nonadherent cells/well) (% viability) ± standard error. Data are expressed as the mean number of plastic nonadherent viable cells from triplicate culture wells.
[d] See Table 2, footnote *d*.

duced during natural *C. burnetii* infections in humans. The results of these studies suggest that the genetics of the individual or the strain of *C. burnetii* are important in determining the outcome of Q fever in humans. It is presumed that viable phase I *C. burnetii* harbors the same components that are extracted with chloroform-methanol since lesions are induced in animals which were either infected or vaccinated by phase I whole cells.

Other virulence factors and the role of antibody in the pathogenesis of Q fever are not understood. Antibodies to both phases I and II *C. burnetii* occur in all forms of the disease (17, 26). The highest levels of antibody occur in chronic disease, suggesting that, in addition to a minor role in protection, antibodies may affect pathogenesis in the form of immune complexes which have been detected in naturally infected humans and in experimentally infected guinea pigs (22, 33). Regardless of the role of antibody, the selective immunological unresponsiveness to antigens of *C. burnetii* may provide a biological advantage so that the microorganisms survive in the granuloma or vegetations on aortic and mitral valves (26). Two isolates from heart valve tissue (26) infected and produced lesions in C57BL/10 ScN mice. However, no marked changes in spleen weight or lymphocyte responsiveness were observed (unpublished results). These results were contrary to those produced by the *C. burnetii* Ohio strain, phase I, laboratory-adapted strain.

Examples of immunomodulation by phase I WCV are the granulomatous hypersensitivity noted in liver, bone marrow, and dermis (2, 30, 31). Recently, Ascher et al. (2) induced dermal granulomatous hypersensitivity with the phase I WCV by using an experimental guinea pig model for *C. burnetii*. In this model, phase I WCV induced epithelioid cell infiltration, multinucleated giant cells, leukocytoclasis, and collagen deposition (2). When CMRV was tested, it induced delayed-type hypersensitivity without granuloma formation and elicitation of antigenic lymphocyte proliferation activity (4). Inferentially, these studies suggest that interacting components extracted by chloroform-methanol from phase I WCV may be considered as virulence factors which have adjuvant-active properties associated with anti-phase I response (Table 2), deleterious tissue reactions represented by hepatosplenomegaly and liver necrosis (32), induction of lymphocyte hyporesponsiveness and negative modulation (9), and elicitation of granulomatous hypersensitivity in guinea pigs (4).

Activities associated with phase I WCV may be somewhat analogous to the clinical and experimentally induced immunologic unresponsiveness described for other microorga-

TABLE 6. Effect of *C. burnetii* fractions on C57BL/10 ScN spleen weights and splenic lymphocyte responses to ConA, PHA, and PWM

Mouse treatment[a]	Spleen wt (mg) (mean ± SE)	[^{3}H]thymidine incorporation in:									
		Nonstimulated cultures (cpm)[b]	Cultures stimulated with:								
			ConA			PHA			PWM		
			cpm	SI[c]	% Supp[c]	cpm	SI	% Supp	cpm	SI	% Supp
Saline	94 ± 4	6,430 ± 274	180,256 ± 3,124	28.0		59,745 ± 1,583	9.3		84,235 ± 7,850	13.1	
WCV	799 ± 102	8,014 ± 1,192	11,756 ± 2,937	1.5	95	6,972 ± 828	0.9	90	10,612 ± 2,559	1.3	90
CMRV	92 ± 5	5,618 ± 322	196,286 ± 2,738	35.0	−25	61,669 ± 598	11.0	−18	91,651 ± 1,143	16.3	−24
CME	94 ± 3	7,737 ± 1,006	192,734 ± 4,333	25.0	11	65,513 ± 7,166	8.5	9	87,071 ± 9,122	11.3	14
CMRV + CME	528 ± 39	8,783 ± 384	62,580 ± 10,410	7.1	75	9,232 ± 754	1.1	88	16,585 ± 2,469	1.9	85

[a] Three mice in each group were given a single 0.5-ml injection i.p. of saline containing 100 μg of *C. burnetii* fractions and assayed for mitogenic responsiveness 2 weeks later.
[b] Table 4, footnote *c*.
[c] For an explanation of SI and % Supp, see Table 2, footnotes *d* and *e*.

TABLE 7. Effect of *C. burnetii* fractions on the protection of C57BL/10 ScN mice against a lethal challenge of viable phase I *C. burnetii*

Mouse treatment[a]	% Survival[b]	*P* value[c]	Mean survival time (days ± SE)[d]
Saline	0		9.0 ± 0.6
WCV	50	0.0325	11.4 ± 4.6
CMRV	80	0.0007	18.1 ± 9.3
CME	0		8.9 ± 0.9

[a] Mice were given a single 0.5-ml injection i.p. of saline containing 100 μg of WCV, CMRV, or CME and challenged 14 days later with a 0.5-ml injection i.p. of phase I Ohio strain (7×10^{10} PFU per mouse).

[b] Mice in groups of 10 were observed daily for 30 days.

[c] *P* values with Fischer's exact test (two tail). Comparisons were made between saline and WCV and saline and CMRV. The difference between WCV and CMRV gave a *P* value of 0.175.

[d] Mean survival time of dying mice plus or minus standard error after challenge.

nisms (6, 28). *C. burnetii* can modulate the immune response in several ways, including potentiation (14–16) and immunosuppression (9, 17). Adverse properties of phase I *C. burnetii* may be similar to the biological activities of cell wall components of the mycobacteria (6, 7, 24). The immunological unresponsiveness elicited by the mycobacterial products (6, 24) has been correlated with pathogenesis and especially with the ability of mycobacteria to evade the action of lysosomal enzymes. A key antimicrobicidal mechanism of *C. burnetii* is the adaptation of this obligately intracellular acidophilic bacterium (12) to replicate in the phagolysosome of immune or nonimmune host cells (5).

Induction of immunoregulatory mechanisms to function in pathologic and suppressor modes may be initiated by the composition of the antigenic mass. Unique determinants present on phase I *C. burnetii* may induce specific suppressor cell functions that regulate the activity of lymphocytes to mitogens and antigens. No unique epitopes of *C. burnetii* which induce this immunological unresponsiveness have been identified by others. We show here that phase I *C. burnetii* induce immunological hyporesponsiveness and negative modulation of lymphocytes to mitogens and recall antigens. The extraction or disassociation of a complex from phase I *C. burnetii* by chloroform-methanol extraction yields an efficacious CMRV. Reconstitution of CMRV with CME restored the pathogenic nature of the CMRV to that of the phase I WCV. The CMRV contains proteins, phosphorus, neutral sugars, KDO-like compounds, fatty acids, and phase I lipopolysaccharide (LPSI) (1). Although LPSI is important in the development of immunity (5; unpublished data), it is also considered to be a major determinant of the virulence of phase I *C. burnetii*. However, we show that the presence of LPSI in CMRV does not induce the deleterious reactions and suppression of in vitro lymphocyte proliferation. In a previous study, phase II WCV or infection did not induce lesions or suppression of in vitro lymphocyte proliferation (9). It appears, therefore, that some other components of phase I *C. burnetii* are important determinants of the induction of immunopathological reactions; however, adverse reactions cannot be assigned solely to LPSI. These results indicate that certain phase I cells contain specific determinants of a suppressive complex which are diassociated by chloroform-methanol extraction. The CME contains specific or nonspecific hydrophobic components which are required to reconstitute with phase I CMRV to produce an active suppressive complex. Studies are underway to fractionate the CMRV and CME so that the interacting molecules can be purified and characterized as to immunogenicity and pathogenicity.

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