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Biological and Immunological Properties of *Coxiella burnetii* Vaccines in C57BL/10ScN Endotoxin-Nonresponder Mice

JIM C. WILLIAMS* AND JOHN L. CANTRELL

Laboratory of Microbial Structure and Function, Rocky Mountain Laboratories, Department of Health and Human Services, National Institute of Allergy and Infectious Diseases, Hamilton, Montana 59840

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Vaccines prepared from Formalin-killed whole cells of *Coxiella burnetii* (Ohio strain) or from chloroform-methanol residue (CMR) and extract (CME) of such cells were examined for biological and immunological properties in male C57BL/10ScN endotoxin nonresponder mice. Vaccines containing killed whole cells induced a high incidence of gross pathology, as evidenced by liver necrosis, significant splenomegaly, and significant hepatomegaly in mice. The degree and onset of these pathological changes were directly and inversely proportional, respectively, to the dosage of killed organisms administered. Conversely, no splenomegaly, hepatomegaly, or liver necrosis were observed in mice inoculated with CMR or CME. Moreover, killed whole cells were lethal for mice at dosages of 150 to 1,200 μg, whereas no deaths were seen in animals given 1,200 μg of CMR. In addition, antibodies against phase I and phase II antigens of *C. burnetii* were detected in the sera of mice inoculated with either whole cells or CMR. Enhanced blastogenic response of splenic lymphocytes was observed when animals were vaccinated with killed whole cells and CMR but not with CME. Moreover, 80 to 90% of mice inoculated with 300 μg of the CMR were protected against a lethal challenge of viable rickettsiae, whereas only 50% of the animals given 300 μg of killed whole cells were protected. Treatments with CME were essentially without value, since no antibodies were detectable and no significant protection was elicited. Collectively, these results show that, although killed whole cells induced immunity in C57BL/10ScN mice, they induced deleterious tissue reactions, whereas CMR, which also induced immunity, was essentially nondeleterious, based on the parameters employed. These observations suggest that the chloroform-methanol–extractable component(s) is implicated in the deleterious tissue reactions and that the phase I and II antigens may not be involved in the induction of the pathology observed in C57BL/10ScN mice.

Previous studies have shown that vaccines prepared from suspensions of killed *Coxiella burnetii* (the etiological agent of Q fever) were effective in eliciting humoral immunity in both humans and animals (17, 20, 21) and were effective in protecting guinea pigs against an infectious dose of organisms (36). Although immunity was elicited with Q fever vaccines, several reports have demonstrated that such vaccines induce severe local and occasional systemic reactions (21). Stoker (37) reported severe local reactions in the form of abscesses and fistulas in vaccinated patients. Similarly, Smadel et al. (36) observed severe local reactions in patients and in guinea pigs inoculated with Q fever whole-cell vaccine. Moreover, they reported that the vaccine was pyrogenic for both humans and guinea pigs. Other deleterious effects from whole-cell vaccines have been shown to include enlargement of the spleen and perisplenitis in guinea pigs (39), skin lesions in rabbits (1), and death in actinomycin D-treated mice (34). In a previous study (10), we showed that administration of Q fever whole cell vaccine to C57BL/10ScN mice resulted in marked and chronic suppression of the normal proliferative response of host spleen cells to various phytomitogens. Thus, the adverse effects observed with whole-cell vaccines emphasize the need for additional studies with fractions isolated from *C. burnetii* by various extraction procedures that possess a protective antigen(s) without producing undesirable biological and immunological tissue reactions.

In the present study, experiments were designed to evaluate the effects of fractions obtained by chloroform-methanol (CM) extraction of killed whole cells on the induction of gross pathological responses, as determined by splenomegaly or hepatomegaly or both, and on the induction of immunity, based on blastogenic response of splenic lymphocytes, production of phase I and phase II antibodies, and protection

against *C. burnetii* infection. Our results showed that suspensions of killed whole cells induced severe splenomegaly, hepatomegaly, and liver necrosis, whereas these pathological changes were significantly reduced or were absent in animals treated with the residue of CM-extracted organisms (CMR). In addition, animals immunized with CMR had high levels of antibodies and resisted a lethal challenge of viable *C. burnetii*. Conversely, no humoral immunity or protection was observed in mice inoculated with the CM extract of whole cells (CME).

MATERIALS AND METHODS

Mice. C57BL/10ScN, endotoxin nonresponder (9, 22), male mice (5 to 10 mice per experimental group) were obtained from the specific pathogen-free production colonies of the Rocky Mountain Laboratories, Hamilton, MT. All mice were 8 to 10 weeks old and weighed 18 to 20 g at the time of inoculation. Mice in an individual experiment were preweighed and varied by ±1 g from each other.

C. burnetii. All studies reported here were performed with suspensions of *C. burnetii*, Ohio strain, phase I. Seed pools of *C. burnetii* (third egg passage) were prepared as 50% (wt/vol, yolk sac per milliliter) suspensions in brain heart infusion (BHI) broth, shell frozen, and stored at −70°C. Rickettsiae from yolk sacs of the fourth egg passage were purified by isopycnic centrifugation in Renografin density gradients (40). The purified organisms were treated with a 1% formaldehyde solution for 24 h at room temperature and dialyzed against 9 liters of sterile demineralized and distilled water at 4°C. The cells were lyophilized and used as whole cells or extracted with CM. The number of organisms and PFU per milligram (dry weight) of *C. burnetti* cells were determined by the method of Ormsbee et al. (28). A 1-mg amount of purified whole cells contained 3.78×10^{10} organisms and 1.8×10^{10} PFU, numbers which agree with previously published results (28, 40).

To determine whether the 1% formaldehyde treatment effectively killed *C. burnetii* cells, a seed pool containing 10^{11} organisms per ml was treated as above and inoculated (0.4 ml) into the yolk sac of 6-day-old embryonated eggs. No egg deaths were observed after 15 days of incubation, and no organisms were recovered after two blind egg passages.

Preparation of chloroform-methanol–extracted *C. burnetii*. Lyophilized *C. burnetii* whole cells were refluxed with a CM azeotrope of 4:1 which had a boiling point of approximately 53.5°C. To 100 ml of CM was added 150 mg of whole cells, and this suspension was refluxed 6 to 8 h. After cooling overnight, the cellular material was separated from the solvent by filtration through Whatman no. 1 filter paper disks. The residue on the filter was mixed with 100 ml of CM and refluxed 6 to 8 h. This procedure was carried out three times, and the CMR and pooled filtrate were dried separately. This procedure gave recoveries of 98.8%, with 78.1% as particulate material (CMR) and 20.7% as extractable components (CME). Endotoxicity of the CME was estimated by protocols described by Milner and Finkelstein (24). The data are reported as the median lethal dose for 11-day-old chicken embryos inoculated intravenously.

Susceptibility of C57BL/10ScN mice to Q fever. Groups of 10 mice each were inoculated intraperitoneally (i.p.) with 0.5 ml of decimal dilutions of viable *C. burnetii* cells (1.4×10^{11} PFU/ml) suspended in BHI broth or phosphate-buffered saline–0.15 M NaCl–0.01 M NaH_2PO_4-Na_2HPO_4 (PBS). Control mice in each experiment were inoculated with 0.5 ml of BHI broth or PBS. At 30 days after the administration of rickettsiae, the surviving animals were bled through an incision in the ventral tail artery, and the sera were pooled for each group. Surviving mice were killed by chloroform anesthesia, and their spleens were harvested and weighed.

Effect of whole cells, CMR, and CME on spleen and liver weights. In each experiment, groups of five C57BL/10ScN mice, each weighing 20 ± g, were inoculated i.p. with various dosages of rickettsial agents. Dosages of the lyophilized whole cells or CMR were based on the dry weight of material suspended in 0.2 ml of PBS. Suspensions of CME, which was lipoid in nature, were prepared by placing 0.42 ml of CM (4:1) solution containing 10 mg of extract per ml in a sterile glass vial. The solvent was evaporated with a light stream of nitrogen. PBS (2.8 ml) was added to the CME, and the mixture was blended in a Vortex mixture to form a suspension that contained 300 μg of extract per 0.2-ml dose. Control mice were inoculated with 0.2 ml of PBS only.

At various times after treatment, spleen and liver weights were measured in control and test animals. In some experiments, the effects of variations in the dosage of the agents administered and time after injection were evaluated.

After the spleen weights were determined, single-cell suspensions of spleens from individual control and test mice were prepared by gentle disruption in sterile Eagle minimal essential medium supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution, using a Kontes-Duall tissue grinder (Kontes Glass Co., Vineland, N.J.). Powdered media and supplements were obtained from GIBCO, Grand Island, N.Y. After two washes in minimal essential medium, a portion of the spleen cell suspension was diluted in 1% ammonium oxalate, and the total number of nucleated cells per spleen was determined by using a Neubauer hemocytometer.

Blastogenic activity of spleen cells from mice given killed whole cells, CMR, or CME. Uptake of $[^3H]$thymidine ($[^3H]$TdR) by spleen cells from mice given injections of *C. burnetii* killed whole cells or fractions was determined by the procedure of Cantrell and Wheat (8). A total of 1 μCi of $[^3H]$TdR (specific activity, 2 Ci/mmol; New England Nuclear Corp., Boston, Mass.) was added to each culture well (6 mm diameter; Milti-Dish Disposotrays, Linbro Chemical Corp., New Haven, Conn.). The concentration of spleen cell suspensions was adjusted so that 5×10^5 cells per 0.2 ml was added to each culture well containing $[^3H]$TdR. The cultures were incubated for 18 h at 37°C in 5% CO_2 in moist air. Cells were collected by using a Multi-Cell Harvestor (Otto Hiller Co., Madison, Wis.), and the extent of the uptake of $[^3H]$TdR was determined by standard scintillation techniques on an LS-9000 counter (Beckman Instruments, Inc., Fullerton, Calif.). For each experiment,

results of triplicate samples from each of five mice per group were expressed as the mean blastogenic indexes ± standard errors of the mean that were calculated from the following formula: blastogenic index = [(mean counts per minute of test spleen cells)/(mean counts per minute of normal spleen cells)].

Protection against *C. burnetii* infection. C57BL/10ScN mice in groups of 10 were given a single i.p. injection of 30 or 300 μg of killed whole cells, CMR, or CME. At 14 or 30 days later, the animals were challenged with 7×10^{10} PFU of viable organisms of the Ohio strain, phase I, of *C. burnetii*. Animals were observed daily for 30 days. Animals were also challenged subcutaneously (s.c.) as described above.

Serology test. Sera from mice given injections of viable organisms, killed whole cells, or CMR and CME were tested for phase I and phase II antibody by the microagglutinin assay of Fiset et al. (11). The antigens employed, phase I (Nine Mile 306GP [cloned in primary chicken embryo cell culture] [ITC]/4EP) and phase II (Nine Mile 306GP/4EP/1GP [isolated 343 days after infection]/3EP, trichloroacetic acid treated) were kindly provided by M. G. Peacock, Rocky Mountain Laboratories. Titers of the microagglutinin assay are given as the reciprocals of the final serum dilution in which macroscopic agglutination was detected. Results are expressed as the arithmetic means of sera samples analyzed in duplicate sets.

RESULTS

Susceptibility of C57BL/10ScN mice to *C. burnetii* infections. Groups of adult C57BL/10ScN mice were inoculated with decimal dilutions of viable Ohio strain, phase I of *C. burnetii* cells (Table 1). A high incidence of mortality (95%), with a short mean survival time (9.6 days), was observed in animals inoculated with 7×10^{10} PFU. The percentage of mice dying of infection decreased with decreasing dosages of rickettsiae. No deaths were observed in groups of mice inoculated with 7×10^5 PFU or less. As determined by the method of Reed and Muench (33), the mouse 50% lethal dose was 2.5×10^8 PFU. Animals injected s.c. gave essentially the same results.

To determine whether viable rickettsiae induced splenomegaly or humoral immunity or both, the mice in the above study that survived an inoculum of viable *C. burnetii* cells were bled for sera, and their spleen weights were measured 30 days after treatment. A significant increase in spleen mass was observed in mice inoculated with 7 to 7×10^{10} PFU of rickettsiae when compared with BHI broth-inoculated control animals. The degree of splenomegaly was roughly proportional to the number of organisms injected. The most striking increase in spleen mass (greater than eightfold) was seen in mice surviving an inoculation of 7×10^9 PFU.

An interesting finding was the detection of necrotic foci, appearing as gross pathological lesions, on the livers of animals given injections of viable rickettsiae (Table 1). The number of mice with liver lesions increased with increasing dosages of organisms. Histologically, the lesions on the livers were areas of severe granulomatous inflammation and necrosis (D. H. Walker and J. C. Williams, unpublished observations). No gross necrosis was detected on the livers of mice 30 days after the administration of 7×10^5 PFU or less. An impression smear of the spleen and liver of each surviving mouse indicated that

TABLE 1. Survival, splenomegaly, incidence of liver necrosis, and antibody titer of C57BL/10ScN mice given varying doses of viable *C. burnetii*, phase I, Ohio strain[a]

No. of organisms injected	No. of animals	% Dead (MST ± SE[b])	Spleen wt (mg) of surviving mice (mean ± SE)[c]	Surviving mice with liver necrosis (%)	Microagglutination titer (log_2; day 30)	
					Phase I	Phase II
7×10^{10}	20	95 (9.6 ± 0.3)	836[d]	100	10	>12
7×10^9	30	77 (14.2 ± 1.4)	1141 ± 83	100	9	>12
7×10^8	30	53 (14.6 ± 1.4)	1044 ± 109	64	10	12
7×10^7	30	20 (13.0 ± 2.0)	953 ± 55	45	10	11
7×10^6	20	5 (13)[e]	749 ± 43	32	10	10
7×10^5	30	0 (>30)	515 ± 22	0	10	10
7×10^3	30	0 (>30)	334 ± 20	0	7	10
7×10^1	30	0 (>30)	223 ± 20	0	7	9
7	20	0 (>30)	250 ± 38	0	5	9
7×10^{-1}	30	0 (>30)	189 ± 25	0	0	0
BHI (control)	29	0 (>30)	132 ± 15	0	0	0

[a] All mice were given a single 0.5-ml injection i.p. of varying doses of organisms. Each group was observed daily for 30 days, at which time the survivors were bled for sera and their spleens were removed and weighed. Animals injected s.c. gave essentially the same results. Pooled results of three experiments.

[b] MST, mean survival time (days); SE, standard error.

[c] By Student's *t* test, for difference with control group. For each group, $P < 0.001$, except the group inoculated with 7 PFU ($P < 0.01$) and that inoculated with 7×10^{-1} (not significant).

[d] Results of a single surviving mouse.

[e] Day of death of a single mouse.

these organs contained large numbers of *C. burnetii* cells (data not shown).

Sera from mice surviving infection with varying doses of rickettsiae were evaluated for phase I and phase II antibodies by microagglutinin assay (Table 1). High titers of both phase I and phase II antibodies were detected in the sera of mice inoculated with dosages of rickettsiae ranging from 7 to 7×10^{10} PFU. The lowest antibody titers correlated with low doses of rickettsiae. Control animals were negative for phase I and phase II antibodies at the time point tested. Animals which showed splenomegaly, hepatomegaly, and phase I and II antibodies contained viable organisms, as evidenced by isolation of *C. burnetii* in chicken embryo yolk sacs. Also, spleen and liver impression smears were positive for *C. burnetii*.

Splenomegaly and blastogenic properties of killed whole cells, CMR, or CME. The effects of a single i.p. injection of 300 μg of either killed whole cells, CMR, or CME on spleen weight and spleen cell blastogenic activity were evaluated in C57BL/10ScN mice at various times after treatment (Fig. 1). The results from three to five experiments, using five mice per group per experiment, were essentially identical and have, therefore, been pooled for convenience. A significant increase in spleen weight (Fig. 1A), with a concomitant increase in the total number of spleen cells (Fig. 1B), and uptake of [^{3}H]TdR by spleen cells (Fig. 1C), was observed in animals inoculated with whole cells when compared with mice given saline. At the peak response (day 14), there was a sevenfold increase in spleen weight and a 23-fold increase in the blastogenic index. By light microscopic examination, spleens from mice inoculated with killed whole cells showed a diffuse accumulation of lymphocytes with increased mitotic activity that was interspersed throughout the organ (data not shown). No increase in spleen weight or total number of cells per spleen was seen in mice given either CMR or CME. However, on day 4 after treatment, a marked increase in the uptake of [^{3}H]TdR was observed with spleen cells from mice inoculated with CMR. At subsequent time points, the blastogenic index of similarly treated animals decreased and approached normal values by day 14. No increase in blastogenic index was observed with spleen cells from mice given CME.

The effect of dosage on splenomegaly, blastogenesis, and incidence of liver lesions with respect to time after treatment was evaluated in mice inoculated with either killed whole cells or CMR. Marked increases in splenomegaly (Fig. 2A), total number of spleen cells (Fig. 2B), and blastogenic activity (Fig. 2C) were observed in animals 7 days after injections of 300 μg and, except on day 18, the degree of splenomegaly

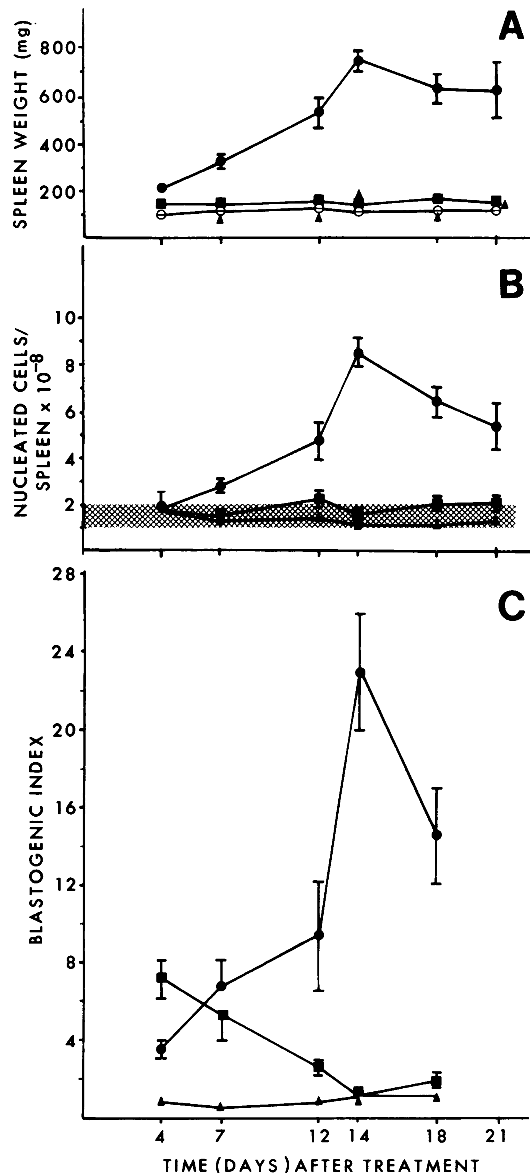

FIG. 1. Biological activity of *Coxiella burnetii* whole cells and subfractions in C57BL/10ScN mice. Comparison of splenomegaly (A), number of spleen cells (B), and blastogenic index (C) in mice given 300 μg of either killed whole cells or fractions from CM-extracted *C. burnetii* cells with respect to time after a single i.p. injection. Controls were given saline. Animals injected s.c. gave essentially the same results. Points represent the means of 15 to 25 mice; bars indicate standard errors. Symbols: ●, whole cells; ■, CMR; ▲, CME; ⊖, saline.

and blastogenesis induced by 100 μg was less than that observed with 300 μg. Increases in spleen weights and blastogenesis were also observed in mice given 10 to 30 μg of whole cells,

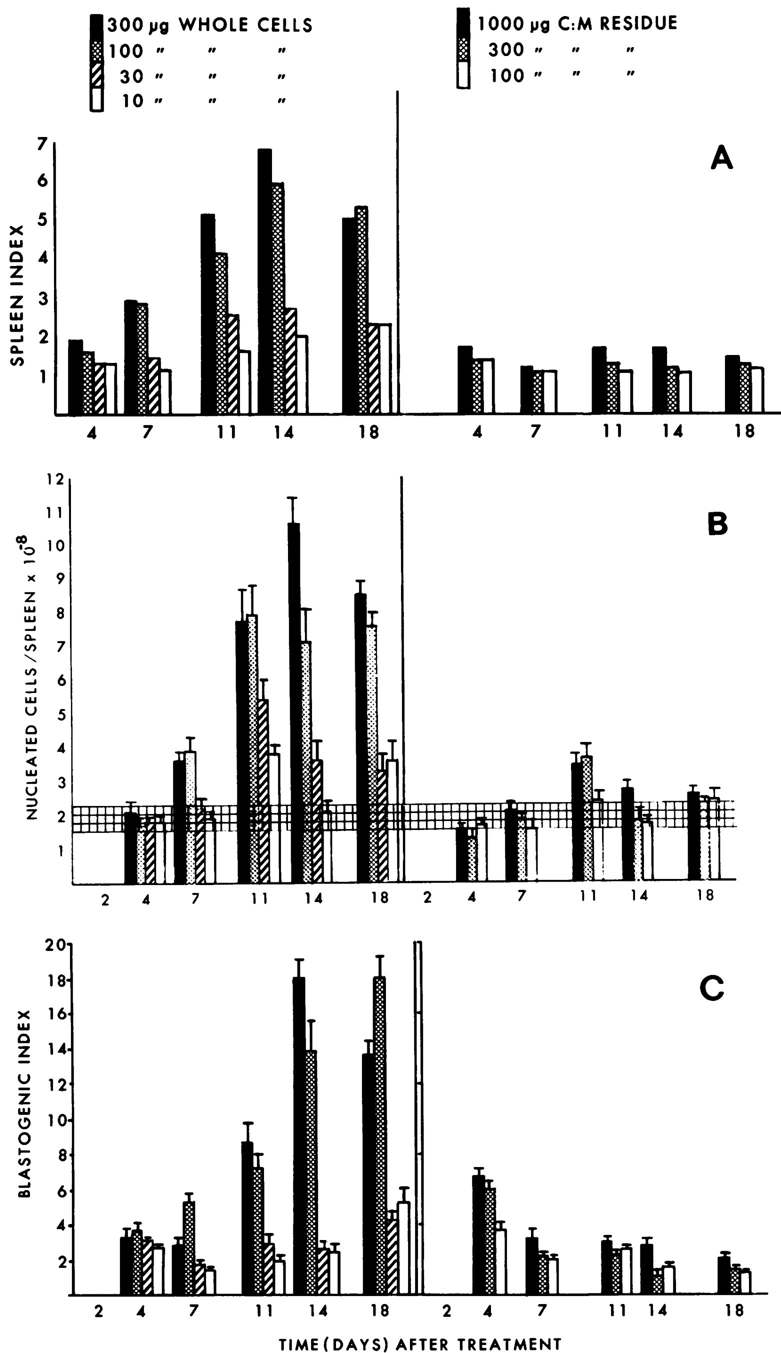

FIG. 2. Biological activity of *Coxiella burnetii* whole cells and subfractions in C57BL/10ScN mice. Effect on splenomegaly (A), number of spleen cells (B), and blastogenic index (C) with varying doses of *C. burnetii* killed whole cells or CMR with respect to time after a single i.p. injection of the agents. Animals injected s.c. gave essentially the same results. Each column represents the mean of five mice; bars represent standard errors.

but the onsets were delayed when compared with higher doses. No increase in spleen weight was observed in animals inoculated with 100 to 1,000 µg of CMR at any of the time points tested. Similarly, except on day 11, no increase in the total number of spleen cells was seen in these animals. In addition, as shown in the previous study, the onset of significant blasto-

TABLE 2. Effect of *C. burnetii*, killed whole cells, and CM residue on the survival of C57BL/10ScN mice

Treatment[a]	Dose (μg)	% Dead	Mean survival time (days) ± SE[b]
Whole cells	1,200	80	27.0 ± 2.1
	600	40	27.1 ± 3.2
	300	40	27.6 ± 3.8
	150	10	26[c]
	30	0	>55
CMR	1,200	0	>55
	600	0	>55
	300	0	>55
	150	0	>55
	30	0	>55

[a] C57BL/10ScN mice (20 animals per group) were inoculated i.p. with microbial components suspended in PBS (0.2 ml per mouse). Animals injected s.c. gave essentially the same results.
[b] SE, standard error.
[c] Day of death of two mice.

genic activity of spleen cells from mice treated with 100 to 1,000 μg of CMR was rapid (day 4) and then decreased thereafter. Animals injected s.c. gave essentially the same results.

Effect of killed whole cells and CMR on mortality of C57BL/10ScN mice. In the foregoing study, occasionally an animal died 15 to 20 days after an injection of 300 μg of whole cells, whereas no deaths were seen in mice given 300 μg of CMR or CME. Therefore, the lethal effects of killed whole cells were compared with those of CMR by inoculating mice with graded dosages (Table 2). A high percentage (80%) of mice died, with a prolonged mean survival time (27 days) after an injection of 1,200 μg of killed whole cells. The incidence of mortality decreased with decreasing dosages of whole cells. Conversely, no deaths were observed in groups of mice given 30 to 1,200 μg of CMR. None of the mice inoculated with 300 μg of CME died (data not shown). In addition, the 50% lethality for chicken embryos of CME was determined to be greater than 200 μg.

To rule out the possibility that viable rickettsiae were still present in the formaldehyde-killed whole cells, 300 μg of this preparation was inoculated i.p. into two 500-g guinea pigs. At 2 weeks after treatment, each guinea pig was killed by chloroform anesthesia, and its spleen was removed and homogenized. Impression smears of the spleens showed intact rickettsiae, as determined by fluorescent-antibody staining (30). The spleen homogenate was inoculated into 6-day-old embryonated eggs. The eggs were incubated at 37°C and observed daily for deaths. No egg deaths or detectable organisms were observed after four blind egg passages. This is the standard technique used to establish the sterility of rickettsiae vaccines (3).

Effect of killed whole cells and CMR on hepatomegaly. To determine whether killed whole cells or CMR have a deleterious effect on the liver, as was previously observed with viable rickettsiae, C57BL/10ScN mice were given injections of 100 to 600 μg of these agents. Significant increases in liver weight were observed on days 8 and 12 after mice were given injections of killed whole cells (Table 3). Conversly, no increase in liver mass was detected in the animal group inoculated with comparable doses of CMR. As previously noted with viable organisms, a high percentage of mice given killed whole cells had grossly visible liver lesions, whereas no lesions were observed on the livers of mice inoculated with

TABLE 3. Effect of *C. burnetii* whole cells or CMR on liver weights of C57BL/10ScN mice

Treatment and dose (μg)[a]	Liver wt (g) per mouse ± SE at following time (days) after inoculation[b]:			No. of mice with liver necrosis/total in group at following time (days) after inoculation (P^c):		
	4	8	12	4	8	12
Whole cells						
600	1.89 ± 0.10	2.09 ± 0.04	2.89 ± 0.08	1/5	15/15 (<0.001)	15/15 (<0.001)
300	1.45 ± 0.06	2.04 ± 0.09	2.88 ± 0.14	0/5 (NS)	8/15 (<0.001)	14/15 (<0.001)
100	ND	1.75 ± 0.08	2.34 ± 0.16	ND	6/15 (<0.001)	12/15 (<0.001)
CMR						
600	1.49 ± 0.03	1.38 ± 0.02	1.44 ± 0.03	0/5 (NS)	0/15 (NS)	0/15 (NS)
300	1.51 ± 0.06	1.40 ± 0.04	1.36 ± 0.04	0/15 (NS)	0/15 (NS)	0/15 (NS)
100	ND	1.34 ± 0.03	1.41 ± 0.03	ND	0/15 (NS)	0/15 (NS)
Saline (control)	1.46 ± 0.04	1.34 ± 0.03	1.35 ± 0.04	0/5 (NS)	0/15 (NS)	0/15 (NS)

[a] All mice were given an i.p. injection of microbial components in PBS (0.2 ml per mouse). Animals injected with CME showed no hepatomegaly or lesions.
[b] Values are expressed as means ± standard error (SE). ND, Not determined.
[c] By Student's *t* test, for difference with control group. NS, Not significant.

CMR or CME (Fig. 3). Based on histological examination, the lesions observed on the livers of mice treated with killed whole cells were areas of severe granulomatous inflammation and necrosis which were similar to those observed with viable *C. burnetii* (D. H. Walker and J. C. Williams, unpublished observations).

Table 4 shows the effect of dosage on the incidence of liver lesions in mice inoculated with whole cells or CMR. The majority (60 to 100%) of mice given 30 to 300 μg of whole cells had detectable liver lesions 11 days after treatment. In addition to a decline in the incidence of lesions, the onset of detectable liver lesions was prolonged with decreasing dosages of whole cells. In contrast, no evidence of liver lesions was observed in mice given injections of 100 to 1,000 μg of CMR.

Antibody response in C57BL/10ScN mice. Table 5 shows the results of comparative serological tests (microagglutinin assay) performed on serial bleedings from mice inoculated with killed whole cells, CMR, or CME. Similar levels of phase II antibodies were detected on day 4 in animals immunized with 300 μg of either killed whole cells or CMR. Slightly lower titers were detected in animals immunized with 30 μg of these subfractions. In animals immunized with 300 μg of whole cells or CMR, low levels of phase I antibodies were first detected on day 7. Similar results were observed in animals inoculated with 30 μg of whole cells, but no development of phase I antibodies was seen in animals immunized with 30 μg of the CMR, suggesting that an adjuvant-like component may have been extracted by CM. In general, phase II agglutinins appeared earlier and were of higher titer than phase I agglutinins in animals immunized with either whole cells or CMR of *C. burnetii*. In contrast, no phase I or phase II antibodies were detected in the sera of mice immunized with the CME.

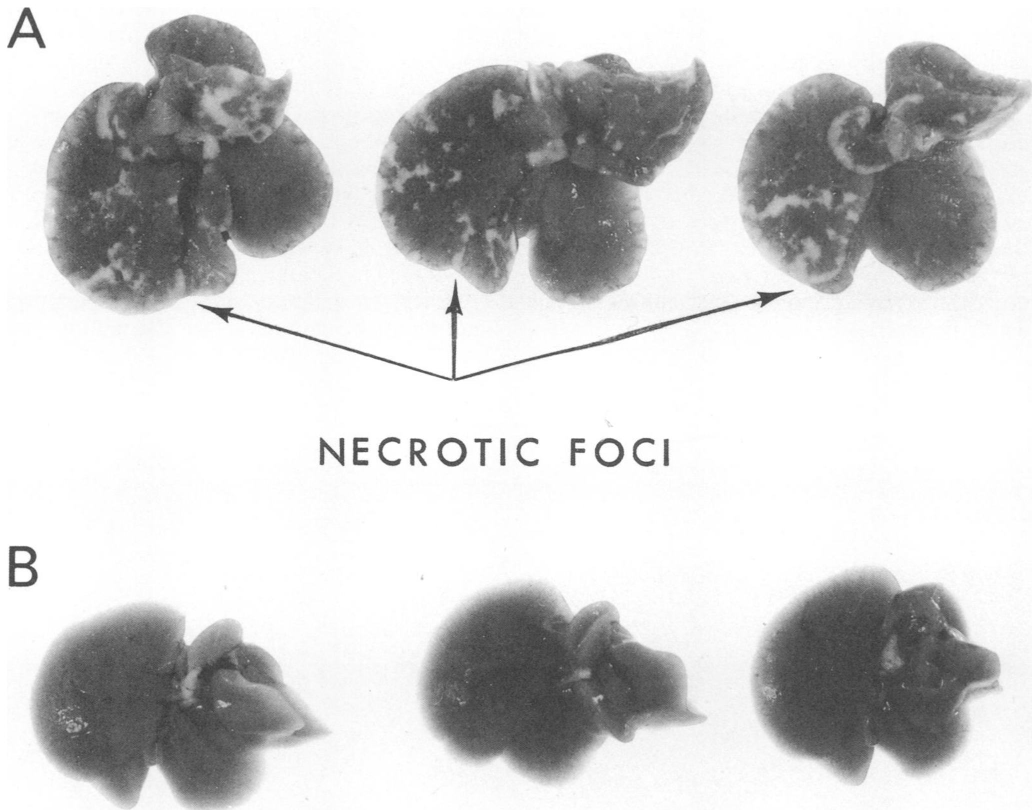

FIG. 3. Typical examples of the relative size and degree of necrosis of livers from C57BL/10ScN mice given injections of 300 μg of either *C. burnetii* killed whole cells (A) or CMR (B). Animals injected s.c. gave essentially the same results. Bright spots on the livers from group B are photographic reflections; no necrosis was observed on these livers.

TABLE 4. Incidence of liver necrosis in C57BL/10ScN mice inoculated with *C. burnetii* whole cells or CMR[a]

Treatment and dose (μg)	% Mice with liver necrosis at following time (days) after treatment:				
	4	7	11	14	18
Whole cells					
300	0	40	80	100	80
100	0	20	100	100	100
30	0	0	60	80	80
10	0	0	0	20	60
CMR					
1,000	0	0	0	0	0
300	0	0	0	0	0
100	0	0	0	0	0
Saline (control)	0	0	0	0	0

[a] C57BL/10ScN mice were inoculated i.p. with microbial components (0.2 ml per mouse). Results are expressed as the percentage of mice with liver necrosis (five mice per group).

Cellular immune response in C57BL/10ScN mice. Since in a previous study we had shown that whole-cell vaccines of *C. burnetii* suppressed the normal mitogenic response to concanavalin A, phytohemagglutinin, and pokeweek mitogen (10), we tested the effect of pretreatment of mice with CMR. These studies, carried out as previously described (10), indicated that splenic leukocytes were not suppressed when mice were pretreated with 300 μg of CMR per mouse over a time course of 7, 14, and 21 days (J. C. Williams and T. A. Damrow, unpublished results).

Protection against *C. burnetii* infections. Finally, it was important to determine whether CMR or CME was efficacious in protecting C57BL/10ScN mice against a lethal challenge of *C. burnetii*. In these studies, mice were given a single i.p. or s.c. injection of either 30 or 300 μg of killed whole cells, CMR, or CME and challenged 14 or 30 days later with 7×10^{10} PFU of viable Ohio strain, phase I of *C. burnetii* (Table 6), a dose previously shown to be highly lethal (95% mortality) for C57BL/10ScN mice (Table 1). Of mice immunized with 30 or 300 μg of CMR and challenged 14 days later, 70 to 90% were protected, whereas only 50% of the mice were protected in a similar treatment protocol with killed whole cells. In animals immunized with 300 μg of CMR and challenged 30 days later, 80% of the mice were protected; however, only 10% were protected when immunized with 30 μg of the CMR. A protection test with mice immunized with 300 μg of killed whole cells and challenged 30 days later was not performed, owing to the death of animals before challenge, but 50% protection was observed in mice inoculated with 30 μg of whole cells. In contrast to these results, only 1 of 10 mice inoculated with 300 μg of CME was protected against a lethal challenge of *C. burnetii*. The route of vaccine administration by the i.p. or s.c. route and subsequent challenge by an alternate route gave the same results, as described above.

Noteworthy is the fact that animals vaccinated with CMR and subsequently given a live challenge or vaccinated with killed whole cells developed splenomegaly, hepatomegaly, and liver lesions (data not shown). More important, multiple vaccinations with CMR did not induce

TABLE 5. Serological response in C57BL/10ScN mice immunized with *C. burnetii* whole cells, CMR, or CME[a]

Treatment and dose (μg)	Microagglutination titer ($\log_2$) at following time (days) after immunization:											
	4		7		14		18		21		32	
	PhI[b]	PhII[b]	PhI	PhII	PhI	PhII	PhI	PhII	PhI	PhII	PhI	PhII
Whole cells												
300	0	9.6	1.3	11	4	11.5	5	10	5.5	12	ND[c]	ND
30	0	7	1	10	3	10	5	10	6	10	8	10
CMR												
300	0	9	1	10	2.3	10	2.5	8	4	9	2	7
30	0	5.5	0	8	0	8	0	7	0	7	0	0
CME												
(300)	0	0	0	0	0	0	0	0	0	0	0	0
Saline	0	0	0	0	0	0	0	0	0	0	0	0

[a] Results are expressed as means of three to five sera samples analyzed in duplicate sets. The error of detection in this test is ± 1 $\log_2$ unit.
[b] PhI, Nine Mile phase I antigen; PhII, Nine Mile phase II antigen.
[c] ND, Not determined, owing to death of mice.

TABLE 6. Effect of killed whole cells of *C. burnetii*, CMR, or CME on the protection of C57BL/10ScN mice against a lethal challenge of viable *C. burnetii*[a]

Treatment and dose (μg)	Challenge time (days)	% Surviving	Mean survival time (days) ± SE[b]
Whole cells			
300	14	50	15.8 ± 5.8
30	14	50	9.0 ± 3.5
300	30	ND[c]	–
30	30	50	8.8 ± 0.9
CMR			
300	14	90	27[d]
30	14	70	9.3 ± 3.7
300	30	80	11,12[d]
30	30	10	10.1 ± 0.5
CME			
(300)	14	10	8.9 ± 0.9
Saline	14	0	9.0 ± 0.6
	30	10	8.8 ± 0.4

[a] All mice were given a single 0.2-ml injection i.p. of microbial components in PBS and challenged 14 or 30 days later with a 0.5-ml injection i.p. of Ohio strain, phase I rickettsiae (7×10^{10} PFU per mouse). Animals injected s.c. gave essentially the same results. Each group was observed daily for 30 days.

[b] Mean survival time of dying mice ± standard error (SE) after challenge.

[c] ND, Not determined, owing to death of mice.

[d] Death time of one or two animals.

these pathological changes. Thus, the gross pathology induced by injection of killed whole cells could not be prevented by previous vaccination of mice with CMR.

DISCUSSION

In animals experimentally infected with *C. burnetii*, susceptibility to infection is usually based upon febrile response, serological conversion, detection of the organism in tissues, or histological evaluation (25, 28). In addition, recent studies have demonstrated marked splenomegaly, hepatomegaly, and necrotic lesions in the spleens, livers, lungs, and hearts of either guinea pigs (12, 29) or mice (6, 32) infected with *C. burnetii*. Similar findings have been observed in the few reported cases of fatal human disease (38). Although both mice and guinea pigs are highly susceptible to infection (50% infective dose, 1 to 2 PFU of *C. burnetii*, phase I, Nine Mile; see reference 28), mortality is rare in both species unless high dosages are employed. Scott et al. (35) recently reported high mortality rates in mice given an i.p. inoculation of 10^8 mouse infective doses of *C. burnetii*. The incidence of death decreased in animals inoculated with 10^6 mouse infective doses. The results reported in this paper support and expand these earlier studies. The data presented in Table 1 clearly demonstrate that the inbred C57BL/10ScN strain of mice is susceptible to *C. burnetii* infection. Although the dosage required to kill mice was large, the mortality (53 to 95%) was directly dependent on the dosage of rickettsiae administered. In addition, based on the mortality rates with serial dilutions, a 50% lethal dose of 2.4×10^8 PFU was established. High levels of phase I and phase II antibodies were detected in the sera of mice inoculated with 7 to 7×10^{10} PFU of rickettsiae (Table 1). The detection of phase I and phase II antibodies in the sera of mice inoculated with 7 PFU is an indication of serological conversion and is consistent with the results of Ormsbee et al. (28), who reported that the 50% infective dose in *C. burnetii* phase I-infected Swiss mice was approximately 1 PFU.

Many studies have shown that vaccines prepared from suspensions of killed whole cells of *C. burnetii* induce humoral immunity against phase I and phase II antigens in both humans (17, 20, 21) and experimental animals (26, 27). In addition, cell-mediated immunity, as measured by delayed hypersensitivity, has been observed in vaccinated guinea pigs (13). Besides these observations, killed whole-cell vaccines have been shown to be pyrogenic for both humans (36) and guinea pigs (4). Moreover, Meiklejohn and Lennette (23) reported severe local reactions in the form of abscess in vaccinated patients. Skin lesions have also been observed in rabbits inoculated with sterile whole-cell vaccines (1, 7). Attempts by other investigators have been made to fractionate *C. burnetii* cells by trichloroacetic acid and phenol; however, the extracts were shown to be pyrogenic in experimental animals (1, 2, 4). Anacker et al. (1) reported that, in addition to causing skin lesions, a trichloroacetic acid extract of *C. burnetii* cells was less potent as an immunogen than killed whole cells. In the present study, experiments were designed to compare and contrast the deleterious effects and therapeutic efficacy of vaccines prepared from killed whole cells with vaccines made from fractions of CM-extracted rickettsiae. Collectively, the deleterious nature of the killed whole cells was clearly a function of the dosage administered, as determined by splenomegaly (Fig. 1 and 2), hepatomegaly (Table 3), incidence of liver necrosis (Table 4), and mortality (Table 2). Conversely, at the dosages tested, no splenomegaly, hepatomegaly, or liver necrosis was observed in mice inoculated with either CMR or CME. More important was the observation that high doses (1,200 μg) of CMR failed to kill C57BL/10ScN mice (Table 2).

These results suggest that the deleterious component(s) was either destroyed or dissociated by CM extraction.

Although the mechanism of splenomegaly induced by *C. burnetii* is unknown, the demonstration of increased uptake of [^{3}H]TdR by spleen cells and of increased mitotic activity in spleens by histological examination suggest that, in part at least, splenomegaly is a direct result of increased cell proliferation and immunological priming. These results may be similar to those of Jerrells et al. (15) and Kishimoto et al. (18), who reported an increase in lymphocyte transformation in vitro to whole-cell antigens from *C. burnetii* with sensitized lymphocytes from human subjects and guinea pigs, respectively, but additional studies are needed to confirm this hypothesis. Although the nature of the component in *C. burnetii* cells responsible for the splenomegaly or lymphocyte proliferation has not been explored, a lipopolysaccharide (LPS)-like substance has been extracted with trichloroacetic acid or phenol (2), which has been shown to be deleterious for mice, pyrogenic for guinea pigs, and to induce hepatomegaly in guinea pigs (for review, see reference 34). Moreover, Brezina et al. (5) reported that extracts prepared by trichloroacetic acid were pyrogenic for guinea pigs and that the neutralization of the pyrogenic effect with *C. burnetii* antibody decreased the protective properties of the extract. Thus, it seems reasonable to speculate that the mechanism of splenomegaly induced by *C. burnetii* cells may be similar to that observed with LPS isolated from gram-negative bacteria (31). Recent studies have shown that the C57BL/10ScN mice used in this study are refactory to the mitogenic and deleterious effects of LPS from gram-negative bacteria, and they are thus classified as endotoxin nonresponders (9, 22). Thus, it appears that LPS is not responsible for inducing splenomegaly in this system, since both killed whole cells and CMR contain equivalent amounts of LPS (J. C. Williams and K. Amano, unpublished results). Alternatively, peripheral lymphocytes may be attracted to the spleen, thus explaining the histological observation of increased accumulation of lymphocytes. In preliminary studies, a marked lymphopenia as measured by total leukocyte and differential counts was observed in the blood of mice inoculated with killed whole cells, whereas mice that were splenectomized before whole cell administration or immunized with CMR had normal numbers of lymphocytes (unpublished results). These observations suggest that the CM-extractable component(s) is responsible for the deleterious tissue reactions and that the phase I and II antigens may not be involved in the induction of the pathology observed in C57BL/10ScN mice.

Based on the parameters employed in this study, the above data indicate that vaccines prepared from killed whole cells cause severe, life-threatening effects in C57BL/10ScN mice, whereas vaccines made from CMR do not. However, an important concurrent question to be answered was which, if any, of the fractions elicit immunity against *C. burnetii* infection. To address this question, a comparison was made between the antibody response in C57BL/10ScN mice immunized with CMR or CME and the response elicited by killed whole cells. Mice immunized with these subfractions were challenged with a lethal dose of *C. burnetii* to determine their state of protective immunity. High titers of phase II antibodies were detected in the sera of mice immunized with 30 or 300 μg of CMR. In agreement with previous studies (11, 16, 17), the production of phase II antibodies was rapid (day 4) and persisted until day 21. The level of phase II antibody from these animals was similar to that detected in the sera of mice inoculated with whole cells. Conversely, the level of phase I antibodies was lower in mice immunized with CMR, as compared with that in animals inoculated with whole cells. Neither phase I nor phase II antibodies were found in the sera of mice inoculated with CME. Finally, the data shown in Table 6 clearly demonstrate the efficacy of whole-cell vaccine in protecting mice against a lethal challenge of *C. burnetii*, a finding which is in agreement with previous studies (27).

That the CMR vaccine induced phase I antibodies at 300 μg but not at 30 μg per animal suggests to us that perhaps CM extraction removes an adjuvant-active component from *C. burnetii*. Reconstitution experiments in which we mixed CMR and CME before vaccinating animals indicated that the deleterious and adjuvant activity could be restored (J. C. Williams and T. A. Damrow, unpublished observations). Importantly, the pathological activity of the killed whole cells or viable *C. burnetii* cells was not abrogated by previous vaccination of mice with CMR. Thus, the CM-extractable component(s) may play a major role in the pathology induced by *C. burnetii*. However, with the exception of animals immunized with 30 μg of CMR and challenged on day 30, the percentage of mice protected with CMR was consistently higher (70 to 90%) than that of animals inoculated with comparable doses of whole cells (50%). Conversely, in the group of mice immunized with 300 μg of CME, only 10% of the animals survived.

In a previous study, we showed that spleen cells from C57BL/10ScN mice pretreated with *C. burnetii* (phase I) whole cells displayed a 20-fold increase in blastogenic activity as determined by increased [^{3}H]TdR uptake (10). Pre-

treatment of mice with whole cells, however, suppressed the normal mitogenic response of spleen cells to concanavalin A, phytohemagglutinin, and pokeweed mitogen (10). In the present study, the protective efficacy of CMR suggested to us that the cellular immune response might not be suppressed. Indeed, preliminary studies indicate that CMR does not induce suppression of normal mitogenic responsiveness. Thus, it appears that the CM-extractable components are responsible for the lymphocyte suppression previously reported (10). Studies in progress are designed to determine the suppressor cell population (10) of proliferating lymphocytes (T or B cells) and to evaluate the role of macrophages, which have been shown to phagocytize *C. burnetii* cells (14, 19), in inducing deleterious tissue reactions. We are currently fractionating *C. burnetii* cells to further describe the chemical composition of the component(s) which induced these adverse and apparently adjuvant-like humoral and cellular reactions. Furthermore, additional studies designed to evaluate the efficacy of vaccines prepared from CMR in protecting guinea pigs from *C. burnetii* infection are in progress.

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