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Efficacy of *Coxiella burnetii* and its chloroform–methanol residue (CMR) fraction against Rift Valley fever virus infection in mice

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Abstract

Strains of *Coxiella burnetii* phase I and II whole cells (WC-I and WC-II) or whole cell fractions were assessed for their potential to induce long-lasting protection in endotoxin-non-responder C3H/HeJ or CD-1 mice against Rift Valley fever (RVF) virus challenge. Among the whole cell fractions, only the chloroform–methanol residue (CMR), administered as a single dose (100 μg per mouse) 24 h before viral challenge, effectively protected 100% of the mice from RVF virus; the CMR of the Ohio strain of *C. burnetii* was not protective. Most of the RVF virus-infected mice treated with other *C. burnetii* cell fractions died, although their times to death varied. Lipopolysaccharide (LPS) associated with CMR preparations used in these studies, did not protect against RVF virus challenge. A single dose of 100 μg of CMR given 24 h before viral challenge completely eradicated 4–5 logs of RVF virus in the serum, liver, spleen, and central nervous system. Compared to several other immunomodulators, CMR was an equally effective antiviral agent. Efficacy of CMR of both Henzerling and Ohio strains disappeared or was marginal when treatment was initiated 2–3 days before RVF viral challenge, even when a second or a third dose of CMR was administered after challenge. A single dose of liposome-encapsulated CMR to

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RVF virus-infected mice extended the range of therapeutic efficacy of this biologically active component of *C. burnetii* to 4 days before infection.

Keywords: Antiviral substance; Rickettsial extract; Immunomodulator; RVF virus

1. Introduction

Use of immunomodulators derived from biological materials is emerging as a rational form of antiviral therapy. Conventional antiviral compounds are designed to inhibit a particular enzyme that may not be universally present among all viruses. In contrast, an immunomodulator may protect against a broad spectrum of viral infections by inducing specific and non-specific antiviral defenses of the host. As a prophylaxis the latter approach offers substantial advantages, because identifying the etiologic agent is not a prerequisite for the commencement of the treatment regimen. Studies of the interactions between viruses and the immune system show that specific and non-specific immune mechanisms can protect the host against viral infections by diminishing viral spread and facilitating more rapid acquisition of specific immunity (Allison and Burns, 1972; Mims, 1982). The importance of each antiviral mechanism strongly depends upon the pathogenesis of the virus in question (Allison, 1974).

From 1983 to 1991, extensive intramural and extramural studies at the United States Army Medical Research Institute of Infectious Diseases evaluated the antiviral potential of large numbers of immunomodulatory compounds including recombinant INF-α-B/D (Gangemi et al., 1989); poly(ICLC) (Kende et al., 1987a, b); recombinant IL-2 (Mead et al., 1991); acridine–HCl and quinolinamine (Kende et al., 1988); poly(ICLC) (Levy et al., 1975); INF-α/β (Morahan et al., 1991); INF-α (Morrill et al., 1991); INF-α/β and acridine–HCl (Pinto et al., 1988); acridine–HCl (Sarzotti et al., 1989); variety of immunomodulators (Sidwell et al., 1988); pyrimidine analogues (Sidwell et al., 1990); INF-α/β (Singh et al., 1989); and poly(ICLC) (Stephen et al., 1977). Poly(ICLC), ampligen, acridine–HCl, quinolinamine, recombinant INF-α/β, pyrimidine analogous (Kende, 1992); poly(ICLC) (Levy et al., 1975); INF-α (Morrill et al., 1989); recombinant INF-α (Morrill et al., 1991); and poly(ICLC) (Stephen et al., 1977) when administered before or after challenge were very effective against arbovirus infections in non-human primates. Marked protection against tumors (Kelly et al., 1976) and a variety of bacterial and viral infections (Waag et al., 1990) was afforded to mice by *C. burnetii* phase I whole cells (WC-I). Among the immunomodulators tested, only WC-I protected the mice completely when given as a single dose 7 days before a lethal viral challenge (Waag et al., 1990). The usefulness of WC-I for antiviral therapy is limited, because administration of WC-I induced significant liver lesions in vivo (Waag and Williams, 1988). Compared to WC-I, the chloroform–methanol-extracted residue (CMR) of WC-I did not cause liver lesions in treated animals, but it retained the antiviral activity of WC-1 (Waag and Williams, 1988).

The purpose of this study was to assess the antiviral activity induced by CMR and other fractions of *C. burnetii*. Identification of a fraction of *C. burnetii* that induces antiviral activity may facilitate the development of natural or synthetic immunomodula-

tors with a long-lasting and broad antiviral capability. In this paper we describe a new Q fever CMR subunit vaccine with antiviral activity against Rift Valley fever (RVF) virus.

2. Materials and methods

2.1. Animals

Female or male, 6–8-week-old inbred, C3H/HeJ endotoxin-non-responder mice (National Cancer Institute, Frederick Cancer Research Facility, Fort Detrick, Frederick, Maryland) or outbred CD-1 mice (Charles River, Inc. Wilmington, Mass.) were used.

2.2. Preparation of C. burnetii, its fractions and CMR vaccine

Phase I (Henzerling, Ohio, and Nine-Mile-Clone 4) and II (Nine-Mile-Clone 4) strains of *C. burnetii* were propagated in yolk sacs of fertile chickens eggs, purified, and inactivated with formaldehyde (Williams et al., 1981). Chloroform–methanol (4 : 1) extraction of formalin-treated and lyophilized phase I whole cells yielded a particulate chloroform–methanol residue (CMR) (Williams et al., 1986) and a CM extract (CME). The final lyophilized CMR phase I Henzerling strain vaccine contained 1% lactose and was reconstituted in saline for injection (Fries et al., 1993). Control mice received saline containing only 1% lactose. CMR (Ohio strain) was further extracted with 100 mM dithiothreitol in PBS at 25°for 1 h (Williams et al., 1986) to produce a cell matrix fraction. The cell matrix fraction was centrifuged at $100{,}000 \times g$ for 30 min, washed with water three times and resuspended in PBS. Lipopolysaccharide (LPS) was isolated from phase I *C. burnetii* Henzerling strain CMR as previously described (Amano et al., 1984).

2.3. Rift Valley fever virus

The Zagazig Hospital 501 strain of RVF virus was isolated during the 1977 epidemic in Cairo, Egypt. The virus was grown in cell culture, and titers were determined by a plaque assay (Meegan, 1979). For quantitation, the virus was inoculated into 24-well culture plates containing 24-h-old, near-confluent Vero cells. Cultures were incubated at 37°C in 5% CO_2 for 60 min to allow adsorption of the virus before the addition of 0.5 ml of overlay medium (0.25% agarose in Eagle basal medium with Earle salt solution, supplemented with 16 mM HEPES [*N*-hydroxyethylpiperaxine-*N'*-2-ethanesulfonic acid], 7.5% heat-inactivated fetal bovine serum, and 5 μg of gentamicin per ml). Cells were incubated further at 37°C until plaques were visible. The tissue cultures were stained with 0.1% crystal violet for plaque counting. In in vivo efficacy studies, 250 PFU ($\sim 10\ LD_{50}$) of virus per 0.1 ml was injected subcutaneously (s.c.).

2.4. Immunomodulators

The following immunomodulators were used: Poly(ICLC) (NIAID, NIH, Bethesda, MD); Quinolinamine (Riker Labs-3M, St. Paul, MN); acridine–HCl (Lederle Laborato-

ries, Pearl River, NY); recombinant human interferon-α-B/D (rHuIFN-α-B/D, Ciba-Geigy, Basel, Switzerland).

2.5. Animal manipulations

Care and use of animals was in full compliance with Animal Welfare Act and with the Guide for the Care and Use of Laboratory Animals, USAMRIID Regulation 360-1. Test animals were housed up to 10 mice/cage. The animal room was lit with fluorescent lights and maintained on a 12-h diurnal cycle. Room temperature and humidity were regulated to 70°F and 50% respectively to avoid extreme fluctuations. Feed and drinking water were provided daily. Fresh bedding and clean cages were provided at least twice a week. Soiled and/or wet bedding was changed as needed. Animals were checked once a day for mortality, and twice a day to ensure proper animal husbandry care. Distress due to bleeding and spleen removal was prevented by using anesthesia with 0.1 ml mixture of xylazin, acepromazine and ketamine (5.0 mg xylazin, 0.1 mg acepromazin, and 40 mg ketamine in 10 ml solution).

2.6. Preparation of liposomal CMR (Fidler et al., 1980)

One ml of CMR dissolved in PBS was added to lyophilized liposomes made of 2 : 1 ratio of L-α-lecithin and phosphatidyl serine prepared by the method of Fidler et al. (1980). The suspension was agitated for 15 min in a water-bath shaker at medium speed at room temperature. The reconstituted liposome suspension contained 100 μg CMR/ml, which was injected s.c. into C3H/HeJ mice.

2.7. Statistical analysis

Estimates of survival distribution function were calculated by using the Kaplan–Meyer product limit method (Lifetest). The differences between treatment effects on survival times were computed by non-parametric Wilcoxon, log-rank statistics (Cary, 1989). Differences between treatment survival proportions were tested using the Fisher's Exact Test.

3. Results

3.1. Evaluation of the efficacy of C. burnetii strain(s) and their fraction(s)

One day before RVF virus infection, groups of 10 CD-1 mice were injected i.p. with 100 μg WC-I, 80 μg CMR, 20 μg CME, 80 μg cell matrix, 100 μg LPS, 80 μg reconstituted CMR with 20 μg CME (all of Ohio strain) or 100 μg WC-II (9-Mile strain). As shown in Fig. 1, RVF viral challenge killed all untreated mice by day 9. WC-I protected 60% of the virus-infected mice, who survived 21 days or longer ($P \leqslant 0.0003$). The cell matrix, LPS, WC-I, WC-II, and the CMR, significantly protected virus-infected mice ($P \leqslant 0.0009$, $P \leqslant 0.0002$, $P \leqslant 0.0004$, $P \leqslant 0.002$, respectively; as

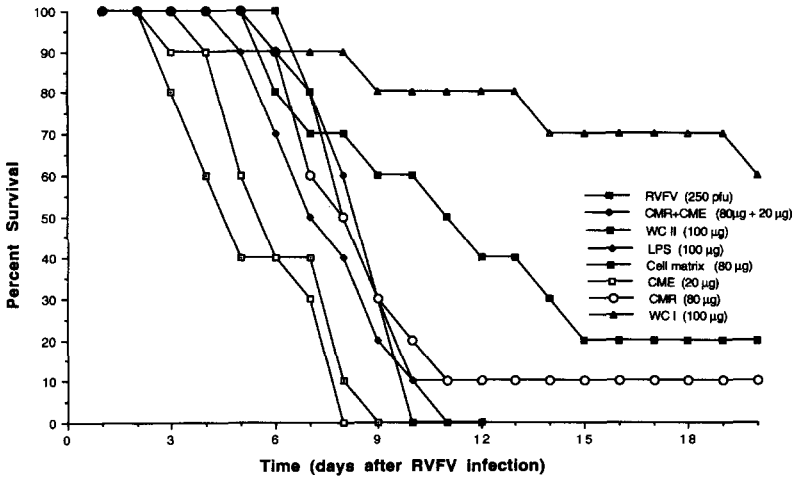


Fig. 1. Efficacy of *C. burnetii* (Ohio strain) fractions RVF viral infection in mice. Mice ($n = 10$) were injected i.p. at day −1 as indicated on the graph, and were challenged s.c. on day 0 with 250 PFU of RVF virus.

calculated by Wilcoxon test) but, these fractions were less effective than WC-I, since with Fisher's two-tail exact text only WC-I protected significant number of the treated mice: $P = 0.01$ or $\geqslant 0.474$ respectively. Neither CME alone nor 20 μg CME combined with 80 μg CMR was effective.

3.2. Comparison of the efficacy of CMR Henzerling and Ohio strains

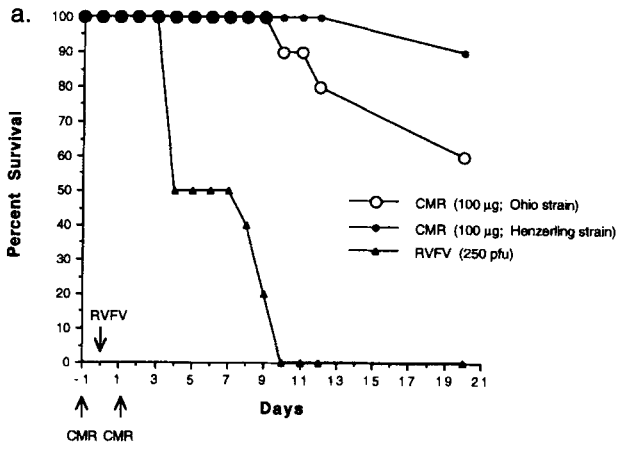
Mice that received 100 μg of CMR from Henzerling or Ohio strains 1 day before and 1 day after being injected with RVF virus, survived (90% and 60%, respectively) 20 days or longer (Fig. 2a). As the time between CMR injections and viral infection increased, the efficacy of CMR decreased (Fig. 2a–c).

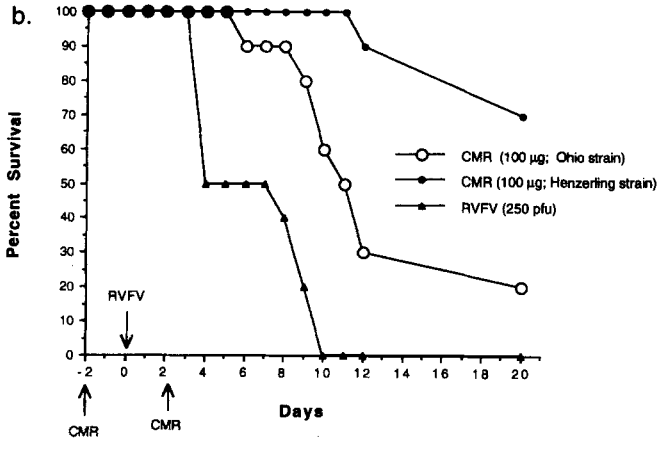
3.3. Effect of treatment spacing on efficacy of Henzerling strain CMR

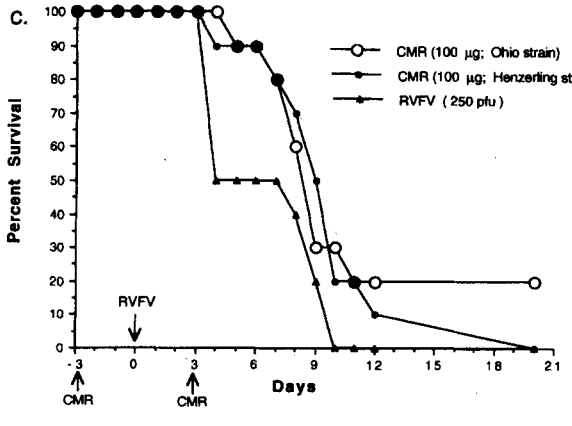
Injecting mice with 100 μg of CMR Henzerling strain at various time intervals from viral challenge (day 0) protected them only when the first dose was administered within 3 days of the viral challenge or less (Fig. 3). Giving mice the third dose of CMR had no effect on the efficacy established with the first two doses (Fig. 2a, b).

3.4. Evaluation of the efficacy of LPS extracted from CMR (Henzerling strain)

C3H/HeJ mice were treated with 100 μg of CMR, 10 μg of LPS (the amount of LPS present in 100 μg CMR), and with 50 μg of LPS 1 day before challenge with 10 LD_{50} of RVF virus (Fig. 4). CMR protected all the infected mice for 21 days or longer ($P \leqslant 0.0005$; calculated by Wilcoxon test). Neither 10 μg nor 50 μg LPS per mouse,


a.
Percent Survival
100
90
80
70
60
50
40
30
20
10
0
CMR (100 µg; Ohio strain)
CMR (100 µg; Henzerling strain)
RVFV (250 pfu)
RVFV
-1 1 3 5 7 9 11 13 15 17 19 21
Days
CMR CMR


b.
Percent Survival
100
90
80
70
60
50
40
30
20
10
0
CMR (100 µg; Ohio strain)
CMR (100 µg; Henzerling strain)
RVFV (250 pfu)
RVFV
-2 0 2 4 6 8 10 12 14 16 18 20
Days
CMR
CMR


c.
Percent Survival
100
90
80
70
60
50
40
30
20
10
0
CMR (100 µg; Ohio strain)
CMR (100 µg; Henzerling strain)
RVFV (250 pfu)
RVFV
-3 0 3 6 9 12 15 18 21
Days
CMR
CMR

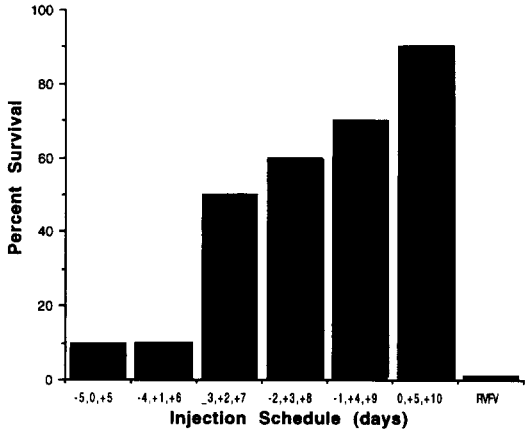


Fig. 3. Efficacy of *C. burnetii* CMR administrated every 5 days at varying time schedules against RVF viral infection. Mice ($n = 10$) were treated i.p. with CMR (100 μg of Ohio or Henzerling strain per mouse) according to the schedules indicated on the graph. Mice were challenged s.c. on day 0 with 250 PFU of RVF virus.

was effective against a viral challenge, although the time to death was increased ($P \leqslant 0.034$ and $P \leqslant 0.0033$).

3.5. Schedule-dependent efficacy of selected immunomodulators against RVF virus infection

Ranking by schedule dependent efficacy, the protection induced with selected immunomodulators in RVF virus-infected mice is summarized in Table 1. These immunomodulators were administered on all possible schedule variations of two doses, when the first dose is given at least 1 day before and the second dose after RVF virus infection. Poly(ICLC) was equally effective when given on day -5 and day 0, or on day -1 and day $+4$ or on all the other variations with 5 day intervals between the 2 doses. CMR was effective only when it was administered on day -1 and on day $+1$. These substances protected all or almost all of the treated mice against RVF viral challenge although, unlike CMR, three of the immunomodulators required less frequent administration.

3.6. Antiviral efficacy of Henzerling CMR

Five mice treated 1 day before RVF viral infection with Henzerling CMR and five untreated, virus-infected control animals were killed and their serum, spleen, liver, and

Fig. 2. Efficacy of *C. burnetii* CMR (Ohio or Henzerling strains) at varying time schedules, against RVF viral infection. 100 μg of Ohio CMR per mouse ($n = 10$) or Henzerling strain was administered i.p. according to the following schedules (a) -1, $+1$, (b) -2, $+2$, (c) -3, $+3$. Mice were challenged s.c. on day 0 with 250 PFU of RVF virus.

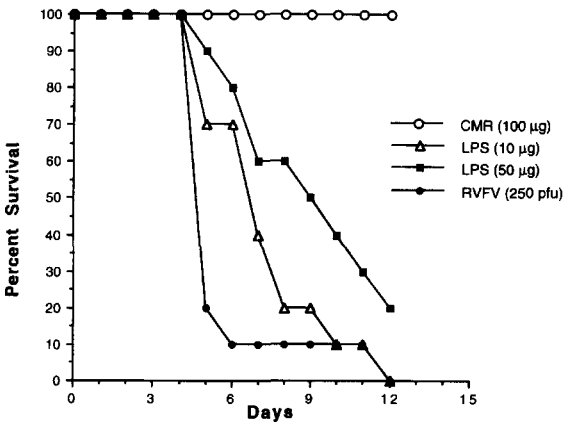


Fig. 4. Efficacy of *C. burnetii* CMR and its LPS against RVF viral infection. Mice ($n = 10$) were treated i.p on day −1 with 100 µg CMR, 10 µg LPS, or with 50 µg LPS per mouse, and were challenged s.c. with 250 PFU of RVF virus on day 0.

brain were removed 4, 48, and 120 h post-infection to determine viral titers. Fig. 5a–d show that 5 days after infection CMR-treated mice had very low viral titers in serum, spleen, and liver, and no virus in brain. In contrast, untreated, virus-infected mice had an average titer of 1.4×10^5 PFU/ml in serum, spleen, and liver, and 3×10^4 PFU/ml in brain.

3.7. Effect of liposome encapsulation on the antiviral efficacy of Henzerling CMR

Groups of mice were treated with free CMR or with liposome-encapsulated CMR 4 days or 1 day before infection with RVF virus. Fig. 6 shows that in contrast to CMR treatment alone, liposome-encapsulated CMR protected mice from lethal RVF, virus challenge ($P = 0.038$ with log-rank and Wilcoxon statistical analysis, and 0.056 with

Table 1
Protection against Rift Valley fever virus infection by select immunomodulators [a]

Compound	Dose	Frequency of administration	Percent protected [b]
Poly(ICLC)	800 µg/kg (i.p.)	every 5 days	100 (56) [c]
Acridine-HCl	1×2.5 mg/kg (oral)	every 4 days	93 (100)
Quinolinamine	50 µg/kg (oral)	every 3 days	75 (92)
rHuIFN-αB/D	10^5 IU [d]/mice (i.p.)	every day	100 (ND)
CMR	100 µg/mice (i.p.)	every 2 days	100 (80)

[a] Mice (CD-1) were injected s.c. with 250 PFU (10 LD_{50}) of RVF virus on day 0.
[b] Percent of mice protected 21 days postinfection.
[c] Numbers in parenthesis indicate the percentage of mice protected against a second challenge with 250 PFU RVF virus, 21 days after the first challenge.
[d] International units.

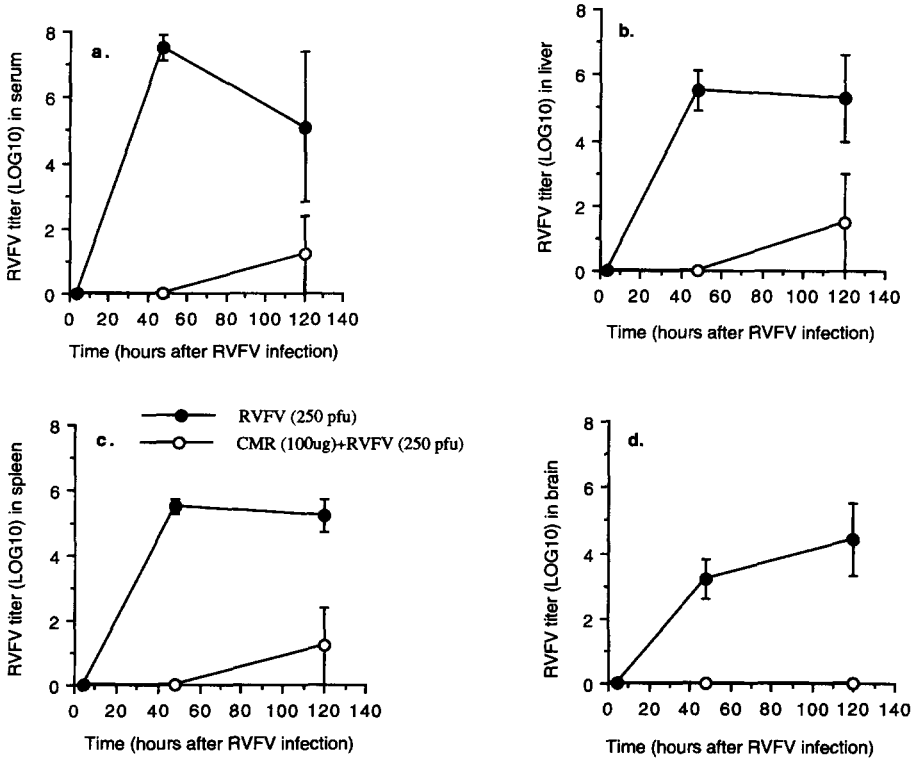


Fig. 5. Antiviral efficacy of *C. burnetii* CMR against RVF virus infection in mice. Mice were treated i.p. on day −1 with CMR (100 μg/mouse). Mice were challenged s.c. on day 0 with 250 PFU of RVF virus. Four, 48 and 120 h after infection the mice were sacrificed and RVF titer in the sera (a), liver (b), spleen (c), and brain (d) were determined by plaque assay. At each time point, five animals were sacrificed in both groups, with the exception of the untreated mice at 120 h, then only 3 mice were alive.

Fisher's one-tail exact test). When liposomes plus CMR were given 1 day before viral infection, there was no difference in the efficacy observed.

4. Discussion

In a previous study, WC-I enhanced the non-specific resistance to EMCV, Banzi virus, and RVF virus infections (Waag et al., 1990) and induced the release of interferon and TNF by spleen cells restimulated in vitro with CMR or WC-I. In the present study, we expanded the previous observation, and provided detailed evidence for the capability of *C. burnetii* WC-I and the CMR fraction to protect against RVF viral infection in mice. This study indicated that CMR protects against RVF virus infection, but the duration or protection was shorter than that induced with WC-I. Compared with WC-I, reconstituted CMR and CME failed to protect the infected mice (Fig. 1). The lack of protective activity of reconstituted CMR and CME may have been due to structural

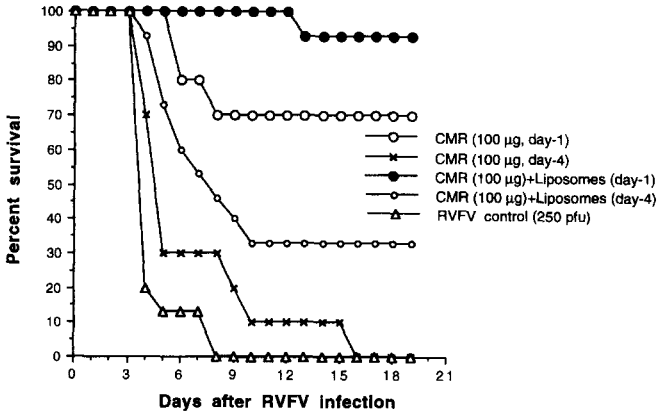


Fig. 6. Efficacy of free or liposome-encapsulated CMR against RVF viral infection. C3H/HeJ mice were treated 1 or 4 days before infection with 100 μg of free CMR ($n = 10$) or 100 μg of liposomal CMR ($n = 15$); untreated control ($n = 15$). Mice were challenged s.c. on day 0 with 250 PFU or RVF virus.

changes caused by the reconstitution which decreased only its antiviral activity, but did not affect the induction of spleen enlargement and liver lesions (Waag and Williams, 1988). Killed WC-1 were lethal for mice at dosages of 150–1200 μg, whereas no deaths were seen in animals given 1200 μg of CMR (Williams and Cantrell, 1982).

CMR does not induce liver lesions, and therefore, it is better suited for human use. A single dose of CMR (Henzerling strain) completely protected the mice against RVF viral infection when it was administered 1 day prior to the challenge. When the treatment was given 2 days prior to the challenge, a second post-challenge dose was required to maintain high efficacy. Spacing the treatment more than two days before and after challenge did not provide protection, which was not improved by a third dose. The biological activity responsible for the efficacy possibly reached a plateau after two doses of CMR when it was spaced not more than four days apart (Fig. 2b), and it was not enhanced by a third dose. LPS constitutes 10% of CMRs weight, and the lipid can act as an adjuvant for CMR. It is unlikely that 10 μg LPS/mice is responsible for the biological activity, since that amount failed to protect mice against lethal RVF virus challenge (Fig. 4). CMR induced several cytokines including IFN-α/β, IFN-γ, IL-1, IL-2, IL-6 and GM-CSF (Zvilich et al., 1994). Only INF-α/β and INF-γ were detectable without in vitro restimulation by CMR. When anti-IFN-α/β and anti-INF-γ antibodies were administered with CMR, the protection was abrogated and the mice died of RVF viral infection (Zvilich et al., 1994). Abrogation of the CMR evoked protection with these antisera is indicative of the major role of INF-α/β and INF-γ in the induction of antiviral state. Similar studies with antiserum to IL-1, IL-2, IL-6 and GM-CSF were not successful (IL-1 and IL-2) with limited studies, or were not done, therefore, at the present time their role in the stimulation of the anti-RVF immune state is not proven. However, participation of these cytokines in the specific and non-specific antiviral response can not be excluded without extensive dose and schedule manipulations with the respective antisera.

The fact that almost no infectious virus was detected in the sera and the organs of infected, CMR-treated mice suggests early clearance of virus by an effective antiviral mechanism stimulated by the immunomodulatory activity of the CMR administered prior or simultaneously to RVF virus infection. The extent of the antiviral activity of CMR, when administered after infection, remains to be determined. CMR and several other immunomodulators (Table 1) protected the mice against a second viral challenge administered 21 days after the first infection. These mice were protected despite the cessation of the therapy. Protection at day 21 was probably due to the presence of a specific antiviral immune response produced by the mice. This immune response developed, despite very low viral titers, thus with a low antigenic stimulus. Although, serum samples were not assayed for antibodies to RVF virus, when CMR was administered with inactivated Venezuelan equine encephalitis virus vaccine (TC 84), the serum IgG ELISA antibodies were elevated 8 folds in 40% of the mice ($n = 10$) 21 days post-immunization. The necessity to administer CMR shortly before or simultaneously with the viral infection to induce protection may be a disadvantage when compared with the protection induced by other immunomodulators listed in Table 1. These substances are very efficacious against three out of four arboviruses families and against DNA viruses as well (Gangemi et al., 1989; Kende, 1992; Kende et al., 1987b, 1988; Levy et al., 1975). Poly(ICLC), acridine–HCl and quinolinamine evoked complete protection when they were injected on every 5, 3, or 2 days schedules respectively, and only rHuINF required daily administration. The requirement that CMR be injected shortly before viral infection may be overcome by employing delivery systems such as oil emulsions, liposomes, microcapsules, or microparticles made of biocompatible/biodegradable polymers. Studies with liposome-encapsulated CMR confirmed this suggestion, since survival rates were enhanced when liposome-encapsulated CMR was administered before infection. Liposomes may deliver the CMR more effectively to macrophages, thereby stimulating secretion of the cytokine cascade, which induces the antiviral activity (Zvilich et al., 1993). Compared with liposome-encapsulated ribavirin, which considerably enhances the efficacy of an ineffective dose against RVF viral infection (Kende et al., 1985) the antiviral efficacy of liposome-encapsulated CMR was moderate. The amount of CMR incorporated into liposomes was not determined, thus the enhanced efficacy could have been achieved with a smaller amount of liposome CMR. Confirmatory studies as well as exploration of other delivery systems for this purpose are in progress.

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