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Characterization of the non-specific humoral and cellular antiviral immunity stimulated by the chloroform–methanol residue (CMR) fraction of *Coxiella burnetii*

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Abstract

Modulation of the immune response by the chloroform–methanol residue (CMR) of phase I *Coxiella burnetii* whole cell was studied in Rift Valley fever virus-infected, or in naive endotoxin-non-responder C3H/HeJ mice. A single dose of CMR completely protected the mice from viral infection. Treating virus-infected mice with antibodies directed against interferons α/β (IFN-$\alpha\beta$) and γ (IFN-γ) eliminated the CMR-induced protection. CMR stimulated the production of high levels of IFN-α/β and 2′-5′-oligoadenylate synthetase activities in sera of the CMR-treated mice. IFN-γ was present in supernatants of cultured spleen cells of CMR-treated, virus-infected mice, but not in their serum. Priming mice with CMR optimized the release of INF-γ, interleukin-1α (IL-1α) and IL-6 from splenocytes in vitro. When stimulated in vitro, IL-2 and granulocyte-macrophage stimulating factor (GM-CSF) did not require in vivo priming for release from cultured spleen cells. Fluorescence-assisted cytometry of CMR-treated mouse spleen cells showed there was a CMR-dependent increase in the percentage of T-cells and Ia-positive

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T-cells. There also was a biphasic increase in the ratio between T_h (L3T4) and T_s (Lyt2) cells. Biological activities stimulated by CMR indicate that CMR is a potent immunostimulant, which may modulate specific and non-specific antiviral responses.

Keywords: *Coxiella burnetii*; Chloroform–methanol residue; Immunomodulator; Rift Valley fever virus

1. Introduction

In spite of the availability of specific antimicrobial agents, viral, bacterial, and fungal infections still represent serious health threats. This is due, not only to resistance of microbial pathogens to antimicrobial agents, but also to the increasing number of patients with compromised immune responses. Enhancing specific and non-specific resistance of the host with immunomodulators derived from microorganisms (Vogels and Vander Meer, 1992) and from other sources, can protect against a broad spectrum of viral infections (Kende et al., 1988).

Coxiella burnetii, the etiologic agent of Q fever, is a potent immunomodulator which can modulate host immune responses positively and negatively. Immune enhancement by components of *C. burnetii* phase I whole cells (WC-I) increases antibody production (Williams and Cantrell, 1982) and stimulates macrophage functions (Kelly, 1977). Mice injected with WC-I had increased resistance to tumors and bacterial infections (Kelly et al., 1976) and resisted protozoan infection (Clark, 1979). However, WC-I also caused pathogenic reactions and immunosuppression in the host. Immune suppression by WC-I and its components results in mitogenic hyporesponsiveness and antigen-specific negative modulation of murine splenic and nodal lymphocytes (Damrow et al., 1985; Waag and Williams, 1988). Inactivating or removing the components responsible for the immunosuppressive pathogenic activity should be possible by dissociating the immunosuppressive complex (ISC) from the WC-I (Nine Mile Ohio, Nine Mile 514 and Henzerling strains) with chloroform–methanol. The biologically active ISC of *C. burnetii* has at least 3 components; one that is extracted by chloroform–methanol (CME), one that is the residue (CMR), and one that attaches the former two components to the cell matrix. When given to mice individually, none of these components appear to have an immunosuppressive activity (Waag and Williams, 1988).

Cytokines play a crucial role in modulating the specific and non-specific immune mechanisms (Koster et al., 1985), and play an additional role in inducing non-specific resistance to bacterial, protozoan, and viral infections (Baron et al., 1991). The possible involvement of cytokines in the biological activity induced by *C. burnetii* components was suggested in a study (Waag et al., 1990) which demonstrated that splenocytes and peritoneal exudate cells from mice injected 14 days previously with inactivated WC-I or CMR, if restimulated in vitro with WC-I or CMR, released IFN and tumor necrosis factor (TNF), respectively.

We studied the immunomodulatory activity of CMR in C3H/HeJ mice infected with Rift Valley fever (RVF) virus, an arbovirus, member of the Bunyaviridae family; RVF virus can cause fatal hepatitis or encephalitis in humans (Easterday, 1965). Immunomodulation is particularly relevant to infections caused by arboviruses, as many of these viral

agents proliferate in macrophages of the liver and in phagocytic cells of other organs. As a result of proliferation of RVF virus in macrophages, the cytokine cascade could be impaired, which leads to dysfunction of the immune response and failure of the host to overcome the infection.

In this study, we studied the biological activity of CMR to understand the mechanism by which this agent augments host resistance to viral infection.

2. Materials and methods

2.1. Animals

Female or male, 6- to 8-week-old C3H/HeJ (endotoxin-non-responder) and CD-1 mice were obtained from the (National Cancer Institute, Frederick Cancer Research Facility, Fort Detrick, Frederick, MD. 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.

2.2. Coxiella burnetii antigens

The phase I Henzerling strain of *C. burnetii* was propagated in yolk sacs of embryonated chicken eggs, purified, and inactivated with formaldehyde (Williams et al., 1981). Chloroform–methanol (4:1) extraction of formalin-treated phase I whole cells yielded the CMR fraction, which was then lyophilized (Williams and Cantrell, 1982). The lyophilized CMR contained 1% lactose and, for injection, it was reconstituted in sterile distilled water. Untreated mice received 1% lactose in sterile distilled water. CMR and placebo preparations were prepared by the Salk Institute (Swiftwater, PA).

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). In efficacy studies, 250 PFU of virus (~ 10 LD_{50}) per 0.1 ml was injected subcutaneously (s.c.).

2.4. Anticytokine antibodies

Anti-IFN-α/β (Lee BioMolecular, San Diego, CA) and anti-IFN-γ antibodies (Genzyme, Boston, MA) were injected i.p. 2–4 h after injection of 100 μg of CMR per mouse, and 24 h before RVF viral challenge.

2.5. Cell supernatants

Splenocytes, isolated from mice injected with 100 μg of CMR 24 h before viral challenge, were prepared as a single-cell suspension and washed with RPMI-1640

medium containing 10% heat-inactivated fetal bovine serum, 1% glutamine, 1% penicillin/streptomycin, 0.1% 2-mercaptoethanol and 2 μg phytohemagglutinin (PHA) per ml. Cells (2×10^5 in 100 μl of medium) were seeded into individual wells of a 96-well cell culture plate. After 0, 24, 48, and 120 h incubation at 37°C under 5% CO_2, supernatants were collected by centrifugation at 1500 rpm for 10 min. Samples collected from cell supernatants were used in studies summarized in Table 1 and Figs. 1 and 2.

Cells were restimulated by adding 10 μg of CMR per ml of medium to 2×10^6 cells per well in 24-well plates. After incubating at 37°C under 5% CO_2, supernatants were collected at times indicated by the experiment. The samples collected were used in studies summarized in Figs. 3 and 6.

2.6. Interferon assay

Spleen cell supernatants and sera from mice were assayed on L-929 mouse cells in 96-well cell culture plates for IFN in a bioassay based on neutralization of vesicular stomatitis virus (VSV) according to published method (Penn and Williams, 1984). The reciprocal of the highest dilution of the test sample that inhibited at least 50% of the cytopathic effect compared with the uninfected cell layers was considered as one unit. The IFN titer was expressed as units per 1.0 ml.

2.7. 2′-5′-Oligoadenylate synthetase activity in serum

The activity was measured by a radioimmunoassay system (Eiken Chemical Co., Ltd., Japan). 2′-5′-oligoadenylate is formed by the catalytic action of IFN-α/β-induced 2′-5′-oligoadenylate synthetase (2-5AS). 2-5AS activity was detected by using ^{125}I-labeled 2-5A as a tracer and a second antibody used to detect the bound/free 2′-5′-adenylate ratio.

2.8. Detection of cytokines

IFN-γ, IL-1α, IL-2, IL-6, IL-10, GM-CSF and TNF-α were determined in sera and in the spleen cell supernatants by using the mouse IFN-γ ELISA (Gibco BRL, Gaithersburg, MD), 'InterTest-1α' and 'InterTest-2x' ELISA (Genzyme, Boston, MA), murine IL-6, IL-10, GM-CSF and TNF-α ELISA kits (Endogen, Boston, MA) respectively, according to the manufacturer's instructions.

2.9. Flow cytometry

Isolated spleen cells (1×10^6) were labeled with monoclonal antibodies (mAbs) directed against Thy 1.2, L3T4, Lyt2, and I-A cell-surface antigens. These mAbs (Becton Dickenson, San Jose, CA) and anti-B220 antibody (Pharmingen, San Diego, CA) were incubated on ice with spleen cells in PBS containing 2% bovine serum albumin and 0.1% sodium azide for 30 min. After incubation, the cells were washed twice with PBS by centrifuging 250 g for 10 min. Cells were resuspended in 0.5 ml PBS containing 0.1% sodium azide. Samples were analyzed by the Becton Dickenson FACS flow cytometer ('FACScan' model) using FACScan Research software.

2.10. Statistical analysis

Differences between treatment survival proportions were tested using Fisher's two-tail exact test.

3. Results

3.1. CMR induction of interferon in uninfected mice

High levels of IFN (1.05×10^4 U/ml) were detected in sera of mice 4 h postinjection of 100 μg CMR (Table 1). Levels peaked 4 h postinjection of CMR. High levels of IFN (9×10^3 U/ml) were also present in supernatants of cultured spleen cells from mice 4 h after CMR treatment (Table 1).

3.2. Induction of interferons by CMR in RVF virus-infected mice

Injecting mice i.p. with 100 μg of CMR 24 h before RVF virus infection induced high levels of IFN-α/β (1.25×10^5 and 4×10^4 U/ml) in sera 4 and 24 h postinfection, respectively (e.g. 28 and 48 h after CMR injection). The high IFN titer vanished by 48 h postinfection (Fig. 1). During 48 h, in the absence of CMR, no INF-α/β was induced by RVF virus alone. With VSV neutralization assay, no IFN could be detected in the supernatant of cultured spleen cells from CMR-treated, RVF virus-infected mice nor from RVF virus-infected CMR-untreated or control mice (data not shown). However, with ELISA assay, 54 units of IFN-γ was detected 72 h after administration of CMR (Fig. 2) in the cultured spleen cells from RVF virus-infected mice (group 2). IFN-γ was not detected in control mice (group 1) and (12 units) was released by the cultured spleen cells from RVF virus-infected, untreated mice (group 3).

Table 1
Stimulation of interferon α/β production by CMR, Henzerling strain in the serum and spleen of C3H/HeJ mice

	Hours			
	4	16	24	48
Serum				
CMR	$1.05\times10^5 \pm 7.5\times10^4$	$5.1\times10^4 \pm 2.0\times10^4$	$3.25\times10^4 \pm 2.25\times10^4$	$5.0\times10^3 \pm 5.00\times10^2$
Control	0	0	0	0
Spleen				
CMR	$9.0\times10^3 \pm 4.9\times10^3$	0	$2.0\times10^2 \pm 3.0\times10^2$	$2.5\times10^3 \pm 9.0\times10^2$
Control	$1.0\times10^3 \pm 1.7\times10^3$	0	$3.0\times10^2 \pm 5.0\times10^2$	$1.0\times10^2 \pm 1.0\times10^2$

Mean ± S.D. units of IFN in 3 experiments in serum and in cultured supernatants from mice infected i.p. with 100 μg CMR or with saline (control).

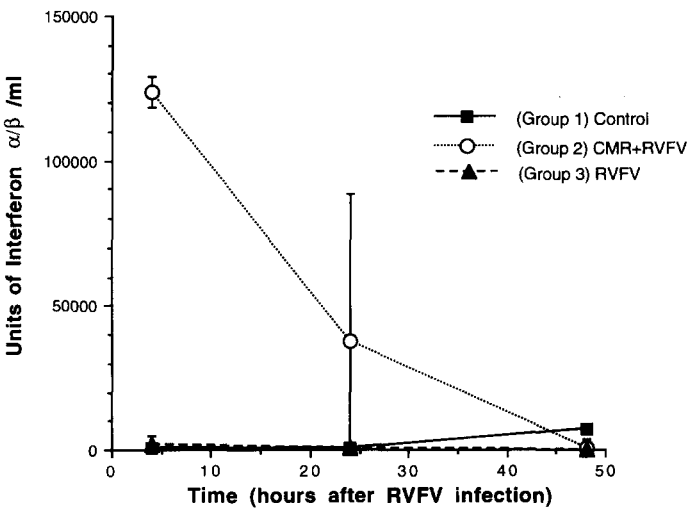

Fig. 1. Stimulation of serum IFN-α/β by CMR in mice ($n = 6$). Group 1, uninfected placebo control; group 2, 100 μg CMR on day -1 and 250 PFU RVF virus s.c. on day 0; group 3, 250 PFU RVF virus s.c. on day 0.

3.3. *In vitro induction of IFN-γ by CMR-stimulated spleen cells*

C3H/HeJ mice were injected i.p. with 100 μg of CMR ($n = 15$) or with placebo ($n = 30$). Twenty-four hours later the mice were killed (see Materials and methods) and

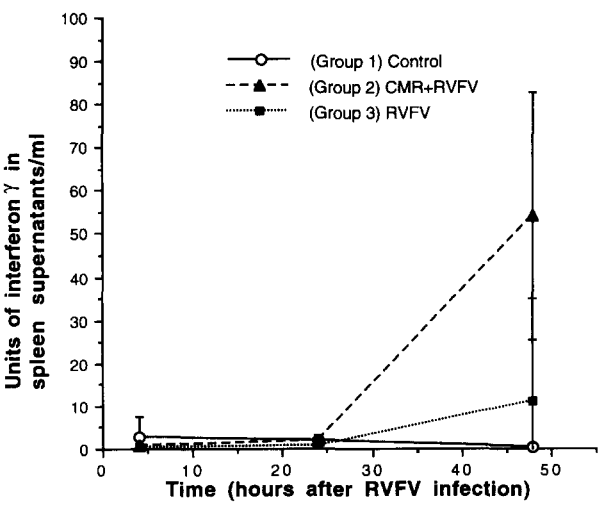

Fig. 2. Stimulation of IFN-γ by CMR in mice ($n = 6$). Group 1, uninfected placebo control; group 2, 100 μg CMR on day -1 and RVF virus s.c. on day 0; group 3, 250 PFU RVF virus s.c. on day 0.

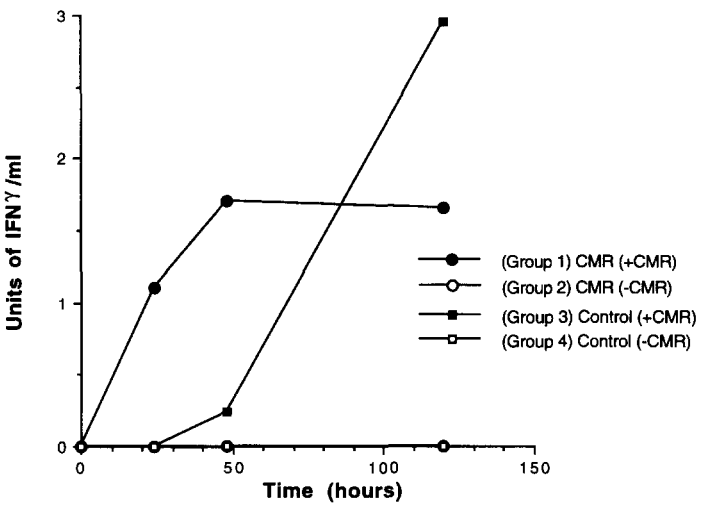

Fig. 3. Stimulation of IFN-γ by CMR. Mice were treated i.p. at day -1 with 100 μg CMR or with placebo. Spleen cells from CMR-treated mice were pooled, cultured, and 24 h later 10 μg/ml CMR (group 1) or medium was added (group 2). Spleen cells from placebo-treated mice were processed by the same procedure (groups 3 and 4). Supernatants were collected at the indicated time points.

pooled spleen cell cultures were prepared. Cell suspensions were incubated with 10 μg CMR per ml. IFN-γ (Fig. 3) was detected in supernatants of restimulated cultured spleen cells which were primed for 24 h in vivo with CMR (group 1), reached a peak level of 300 pg/ml in 48 h after in vitro stimulation (one unit corresponds to 175 pg recombinant mouse IFN-γ as stated in the instruction manual of the kit). In contrast, at 48 h, the IFN-γ level was 43 pg/ml in cultured spleen cell supernatants from control mice (group 3) which were exposed to CMR only in vitro. After 120 h, IFN-γ reached a level of 500 pg/ml. In the cultured spleen cell supernatants from in vivo primed mice, no significant level of IFN-γ was detected when CMR was not added in vitro (group 2). In control mice (without in vivo or in vitro stimulation), IFN-γ was not detectable (group 4).

3.4. Abrogation of IFN-α/β and IFN-γ-related protection by antibodies to IFN in CMR-treated, RVF virus-infected mice

To study the role of IFNs in the protection induced by CMR, mice were injected (i.p) with anti-IFN-α/β and/or anti-IFN-γ antibodies 1–2 h after CMR injection (24 h prior to RVF virus-infection). Anti-IFN-γ antibodies (100 μg/mouse) diminished the CMR-elicited protection from 100 to 70%, while anti-IFN-α/β (3000 U/mouse) reduced the survival rate to 64% (Fig. 4). Antibodies against IFN-α/β (3000 U/mouse) and IFN-γ (100 μg/mouse) in CMR-treated RVF virus-infected mice eliminated the CMR-induced protection almost entirely; only 32% of the mice survived viral challenge ($P = 0.0049$). Survival rate of RVF virus-infected, CMR-untreated mice was 20%.

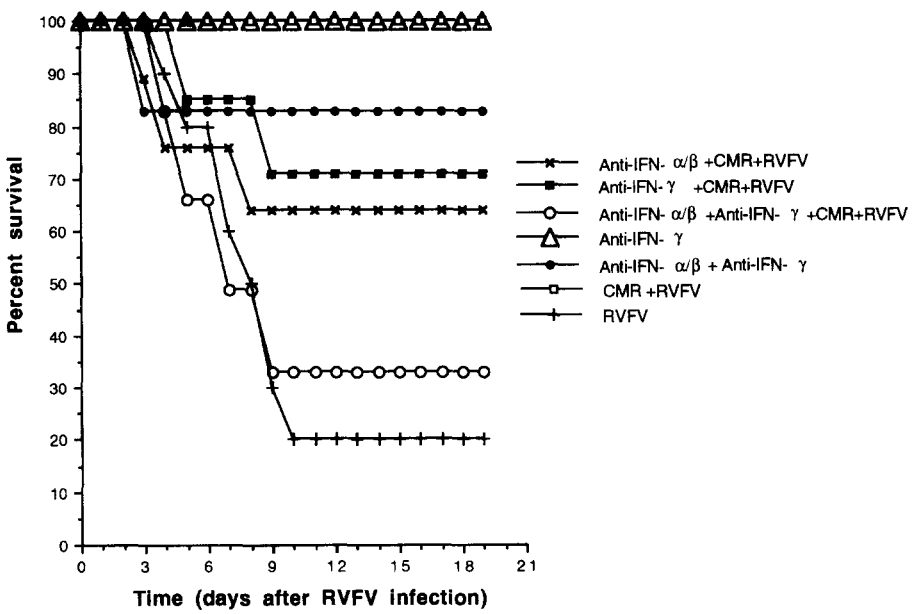

Fig. 4. Abrogation of CMR-evoked protection with anti-IFN-α/β and anti-IFN-γ antibodies in RVF virus-infected mice. Mice were treated i.p. at day -1 with 100 μg CMR and 2–4 h later with 3000 U/mouse anti-IFN-α/β and/or 100 μg/mouse anti-IFN-γ. At day 0 mice were challenged with 10 LD_{50} of RVF virus s.c.

3.5. Stimulation of 2-5AS activity

2-5AS activity was measured in sera of RVF virus-infected and control mice which were killed at various times after infection. As shown in Fig. 5, when virus-infected mice were dosed with 100 μg of CMR per mouse, significant levels of 2-5A (which is formed by the catalytic action of IFN-induced 2-5AS) were detected 24 h after treatment in pooled sera of uninfected, CMR-treated mice up to 3 days after treatment (880 pmol/dl). Similarly high levels (961 pmol/dl) of 2-5A were detected in sera of virus-infected, CMR-treated mice, which peaked between 5 and 6 days, then declined 7 days after infection (Fig. 5).

3.6. Induction of IL-1, IL-6, GM-CSF, IL-10 and TNF-α by in vivo and/or in vitro CMR-stimulated spleen cells

As shown in Fig. 6a, a single dose of 100 μg CMR stimulated IL-1α release in cultured, pooled spleen cell supernatants of mice without in vitro restimulation ($n = 15$): 40, 42, and 80 pg IL-1α per ml, after 24, 48, and 120 h incubation, respectively (group 2). When in vivo-primed, cultured spleen cells were restimulated with CMR (10 μg per 2×10^6 cells) the IL-1α level at comparable time intervals were 37, 50, and 45 pg IL-1α per ml (group 1). Following in vitro stimulation with CMR, after 24 h incubation,

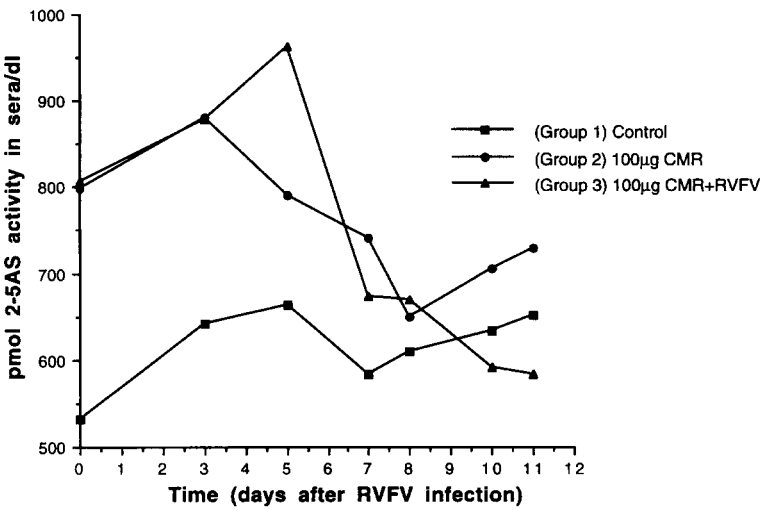

Fig. 5. Detection of 2′-5′-oligoadenylate synthetase activity (2-5A) in the sera of CMR-treated mice infected with 250 PFU of RVF virus s.c.. Group 1, placebo; group 2, 100 μg CMR injected i.p. on day −1 to uninfected mice; group 3, 100 μg CMR i.p. on day −1. RVF virus s.c. on day 0. Sera were pooled at indicated time points and assayed for 2-5A.

significant IL-1α levels (43 pg/ml) could also be detected in supernatants of cultures of spleen cells pooled from control mice ($n = 30$) which were not stimulated in vivo (group 3). However, in this group, the decrease of IL-1α levels was much faster: 12 and 20 pg/ml after 48 and 120 h incubation, respectively. No significant levels of IL-1α could be detected in the supernatants of cultures which were not stimulated with CMR in vivo or in vitro (group 4).

Without in vivo priming, after in vitro stimulation with CMR (Fig. 6b), significant IL-2 levels (40, 1000, and 300 pg/ml) were present in the cultured spleen cell supernatants at 24, 48, and 120 h, respectively (group 3). In vivo priming prior to in vitro restimulation did not augment IL-2 level (group 1). Background IL-2 levels without in vivo and in vitro stimulation at comparable time points were: 38, 70, and 0 pg/ml (group 4), which was not augmented by in vivo priming alone (group 2).

Without in vitro restimulation, no significant levels of IL-2 in the spleen cell culture supernatant obtained from control, or CMR-untreated RVF virus-infected or CMR-treated RVF virus-infected mice, could be detected by ELISA (data not shown).

The highest levels of IL-6 (Fig. 6c) were detected when spleen cells obtained from mice primed in vivo with CMR were restimulated in vitro: 2314 pg/ml 120 h after restimulation (group 1) (Fig. 6c). In vivo priming without in vitro restimulation (group 2) and in vitro exposure without in vivo stimulation (group 3) yielded comparable levels of IL-6: 568 and 615 pg/ml, respectively. No significant levels of IL-6 could be detected in cultured spleen cell supernatants obtained from control mice without in vivo or in vitro stimulation (group 4).

CM-CSF (Fig. 6d) was detected in cultured spleen cell supernatants obtained from mice primed in vivo with CMR and were restimulated in vitro (group 1): 23.9 and 25.7,

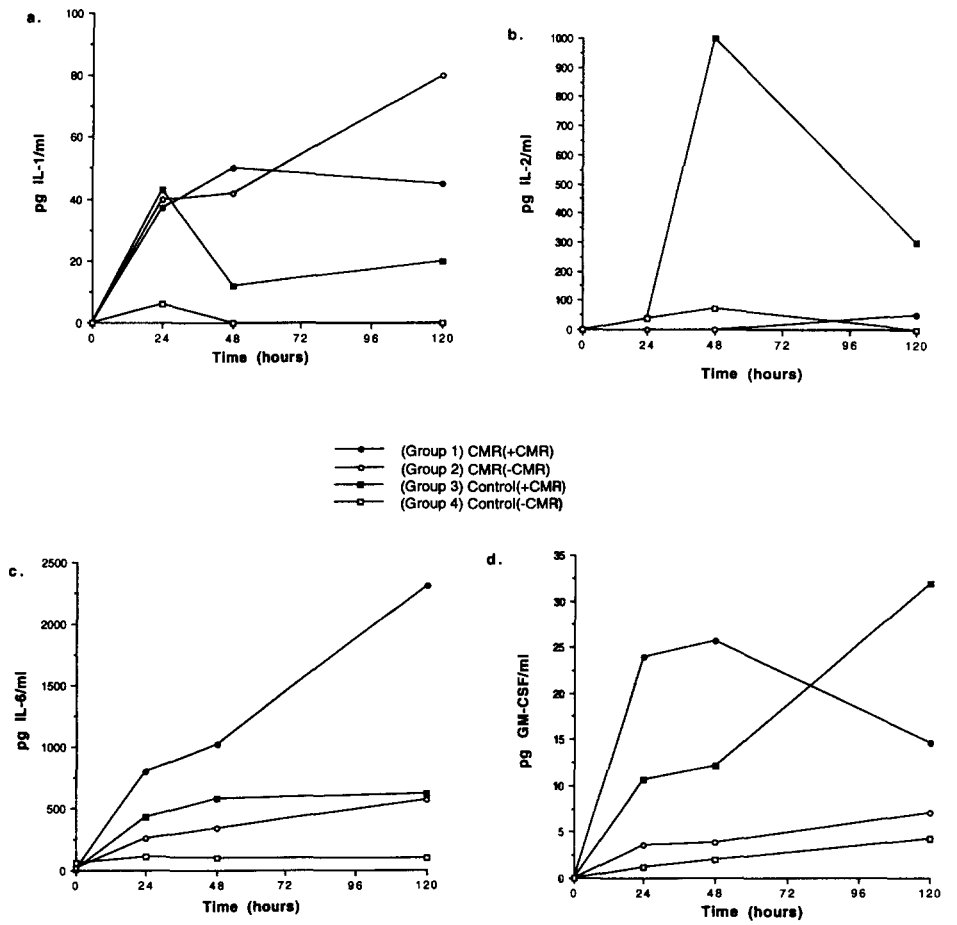

Fig. 6. Detection of IL-1 (a), IL-2 (b), IL-6 (c), and GM-CSF (d) in cultured spleen cell supernatants of CMR-treated mice. Mice were treated i.p. at day −1 with 100 μg CMR or with placebo. On day 0, the mice were sacrificed, spleen cells were pooled, cultures were prepared and reincubated for the indicated time periods with 10 μg CMR/ml (group 1) or with medium (group 2). Spleen cells from placebo-treated mice were stimulated with 10 μg CMR/ml (group 3) or with medium (group 4).

pg/ml peak values after 24 and 48 h, respectively, which decreased to 14.6 pg/ml after 120 h incubation. GM-CSF could also be detected in cultured spleen cell supernatants from control mice after in vitro exposure (group 3): 10.7, 12.1, and 32.0 pg/ml after 24, 48, and 120 h incubation, respectively. In vivo stimulation alone (group 2), or without in vivo and in vitro stimulation (group 4) yielded comparable low levels of GM-CSF: 7.0 and 4.2 pg/ml after 120 h, respectively. No IL-10 or TNF-α could be detected with ELISA (not shown on the figures).

3.7. Analysis of spleen cell surface markers in CMR-treated mice

To characterize possible changes in the lymphocyte population pattern in spleens of CMR-treated and RVF virus-infected mice, cells were stained with monoclonal antibod-

Table 2
Cell surface marker analysis of spleen cells from CMR-treated, RVF virus-infected mice

Cell type (Type of antibody)	% Cells positive to a specific antibody At 72 h				At 120 h			
	Uninfected		Infected		Uninfected		Infected	
	Control	CMR	RVFV	CMR + RVFV	Control	CMR	RVFV	CMR + RVFV
B-cells (B220)	42.7	50.4	43.2	49.2	51	32.5	64.8	32.9
T-cells (Thy 1.2)	12.8	21.6	14.0	23.4	16.9	16.2	15.8	10.7
T_h/T_s (L3T4/Lyt2)	3.4	4.1	3.2	6.0	3.6	3.6	6.3	3.6
Ia-positive	55.0	59.0	52.0	58.3	58.6	41.6	65.8	40.3
Ia-positive T-cells	4.9	7.2	6.1	9.2	7.0	11.2	9.7	5.2

Mice (5 per group) were injected i.p at −24 h with 100 μg of CMR and at 0 h with 10 LD_{50} of RVF virus. At 72 and 120 h after infection, spleens were pooled out, cells isolated and counted. Cells (2×10^6 per ml) were pooled using an equal number of cells from each mice, stained with FITC or PE-conjugated antibodies directed against specific cell surface markers. Spleen cells were analyzed with a 'Becton Dickinson' ('FACScan model') FACS flow cytometer and FACScan research software.

ies directed against specific cell surface markers, and analyzed using a flow cytometer (Table 2). In comparison with untreated uninfected controls, administration of CMR increased the total T-cells by 72 h from 12.8 to 21.6%, an increase of 68%. In the uninfected, CMR-treated mice at this time point, total B-cells, T_h/T_s ratio, Ia-positive or total T-cells did not change considerably. Neither did RVF virus-infection cause considerable shift in these parameters after virus administration. Administration of CMR into RVF virus-infected mice, caused a marked increase in T-cells expressing Thy 1.2 surface marker, in the ratio of T_h/T_s cells and Ia-positive T-cells: 60% (from 14.0 to 23.4%), 87% (from a ratio of 3.2 to 6.0) and 34% (from 6.1 to 9.2%) increases respectively, while values of total B-cells and total Ia-positive cells showed no meaningful changes. At 120 h, the stimulatory effect of CMR on the total T-cells was no longer observable, but, at that time the Ia-positive T-cells increased with 37% (from 7.0 to 11.2%). However, with the exception of the Ia-positive T-cells, the values of all other parameters were lower at 120 h in the uninfected CMR-treated mice. At that time point, in the untreated RVF virus-infected mice, with the exception of total T-cells all other values increased considerably: total B-cells with 36% (from 51.0 to 64.4%), T_h/T_s ratio with 75% (from 3.6 to 6.3), Ia-positive cells with 12% (from 58.6 to 65.8%), and Ia-positive T-cells with 38% (from 7.0 to 9.7%). At 120 h, in the CMR-treated infected mice, the values of all the tested parameters were much lower, either in comparison with the untreated uninfected mice, or in comparison with the infected untreated mice.

4. Discussion

In this study we provided evidence for the capability of CMR (Henzerling strain) to induce in vivo and/or in vitro several immunoregulatory cytokines which are most likely involved in the inhibition of RVF virus proliferation in the host, through generation of non-specific and specific immunity.

Table 3
Summary of lymphokines and interferon production/release pattern in sera and in cultured spleen cell supernatants of C3H/HeJ mice treated in vivo and/or in vitro with CMR

Substance detected	Source	Mode of CMR administration and stimulation			
		In vivo	In vitro	In vivo + in vitro	In vivo (RVFV infected)
IFN-α/β	Sera	↑ [a]	ND [b]	ND	↑
IFN-α/β	Spleen supernatant	↑	ND	ND	↑
IFN-γ	Sera	ND	ND	BDL [c]	BDL
IFN-γ	Spleen supernatant	BDL	↑	↑ [d]	↑
IL-1	Spleen supernatant	↑	↑	↑ [d]	BDL
IL-2	Spleen supernatant	BDL	↑	BDL	BDL
IL-6	Spleen supernatant	↑	↑	↑ [d]	ND
GM-CSF	Spleen supernatant	BDL	↑	↑ [d]	ND
IL-10	Spleen supernatant	BDL	BDL	BDL	ND
TNF-α	Spleen supernatant	BDL	BDL	BDL	ND

[a] ↑, increase.
[b] ND, not done.
[c] BDL, below detectable level.
[d] In vivo priming resulted in a higher and/or faster production/release.

The WC-I of *C. burnetii* can be separated to CME, CMR, cell matrix, and a dithiothreithol-soluble component which binds to the cell matrix (Waag and Williams, 1988). The WC-I of *C. burnetii* was previously shown to enhance resistance to tumor growth, bacterial and viral infections (Kelly et al., 1976; Waag et al., 1990), but WC-I causes pathogenic reactions and suppresses lymphoproliferative responses. In contrast, CMR did not induce immunopathological reactions or suppress mitogenic and antigenic lymphocyte proliferation (Williams and Cantrell, 1982; Williams et al., 1986). In comparison with WC-I, CMR is perhaps better suited for human use since for vaccination of humans, a dose of 100 μg did not cause any local reaction on s.c. administration (Fries et al., 1993). Administration of much higher dose of 240 μg was needed to elicit erythema in humans, but even that dose did not induce granuloma, abscess or systemic reaction. In our study, the same CMR preparation was used. For prospective antiviral human use, no further purification is contemplated at the present time, thus, the dose most likely will not exceed 100 μg, and probably two to three administrations will be required. Therefore, in anticipation of its human use, the biological activities of CMR against RVF virus infection in C3H/HeJ mice were further characterized in this study.

With a few exceptions, the cytokines in the serum of mice are below the level of detection either with the ELISA or the bioassay. Even when spleen cells from in vivo-stimulated mice are cultured in vitro, IL-2, IFN-γ and IL-10 did not attain sufficiently high level for detection (Table 3). Therefore, to augment the level of cytokines by direct and continuous exposure to CMR, the spleen cells from in vivo stimulated mice were cultivated in the presence of CMR.

The CMR stimulated high IFN-α/β response in sera and in spleen cell culture supernatants (Table 1) from infected or naive mice. In CMR-treated RVF virus-infected mice IFN-γ was induced in supernatants of cultured spleen cells (Fig. 2). RVFV alone did not stimulate IFN-α/β or γ during that time. However, a more rapid increase in the level of IFN-γ was seen in uninfected mice, when in vivo priming was followed with in

vitro restimulation (Fig. 3). The higher level of IFN-γ in this case may represent the release of intracellular cytokine by the second signal. The same pattern of production/release was observed for GM-CSF (Fig. 6d). GM-CSF is a glycoprotein secreted by activated T-cells, endothelial cells, fibroblasts, mast cells, B-cells, and macrophages. GM-CSF acts on bipotential stem cells to produce mononuclear phagocytes and granulocytes. Extra macrophages and neutrophils may be required for both antigen processing and for clearing up antigen, following stimulation by other lymphokines such as IFN-γ (Dawson, 1991).

RVF virus is sensitive in vitro to both IFN-α and -β (Kende et al., 1988). RVF virus-infected mice are protected by treatment with recombinant IFN-α or IFN-α/β inducer and macrophage activator (Canonico et al., 1984). Abrogation of the CMR evoked protection with anti-IFN-α/β and anti-IFN-γ antibodies (Fig. 4) is indicative of the major role of these cytokines in the induction of an antiviral state.

CMR contains proteins, phosphorus, neutral sugars, ketodeoxy-octanic acid (KDO), fatty acids, and LPS (Williams et al., 1986). KDO indicates the presence of LPS, which is about 10% of the total weight. That amount of LPS does not appear to contribute to the protective activity of the CMR as determined by its inability to protect RVF virus-infected mice (Zvilich et al., 1995).

The mechanism of IFN-α/β potent antiviral action involves the induction of specific cellular proteins above the pre-existing levels. One well-characterized IFN-induced protein is 2-5AS. Once this enzyme is activated by double-stranded RNA, it generates from ATP, 2′-5′-linked oligoadenylate (2-5A), which in turn activates a latent RNase that cleaves single-stranded RNA at specific sites (Penn and Williams, 1984). Measuring 2-5AS is advantageous as opposed to measuring IFN-α/β since IFN level in the serum decreases rapidly, while the level of 2-5AS remains elevated in cells for several days (Schattner et al., 1981). In this study, extracellular 2-5AS activity could be detected in sera of CMR-treated mice, and in sera of CMR-treated RVF virus-infected mice (Fig. 5). The significance of measuring 2-5A in the serum is supported by the fact that values of 2-5AS activity in serum has similar values and kinetics as those in peripheral blood monocytes (Penn and Williams, 1984).

IFN-γ inhibits viral, protozoan and bacterial infections via activation of bactericidal systems of macrophages and cytotoxicity of NK cells and by interacting with other cytokines (Koster et al., 1985). The involvement of natural killer cells in regulating certain viral infections is documented (Baron et al., 1991). IFN-γ also induces cytolytic activity in LAK and CTL cells, regulates IgA and IgM transport and enhances their secretion by resting B-lymphocytes (DeMaeyer and DeMaeyer-Guignard, 1988). Furthermore, IFN-γ enhances IL-1α production by macrophages (Baron et al., 1991). Macrophage-produced IL-1 can induce IL-2 which in turn induces IFN-γ or IFN-β (Le et al., 1986). Following in vivo stimulation with CMR, higher levels of IL-1α were detected, which did not depend on in vitro restimulation of spleen cells. In vitro stimulation alone resulted in high IL-1α levels which existed for 24 h only. Thus, in vivo pre-exposure of C3H/HeJ mice to the CMR, optimized the in vitro release of IL-1α from the splenocytes.

Contrary to IL-1α induction, IL-6 was induced by a single in vivo or in vitro CMR signal. However, double signaling (in vivo and in vitro) yielded a much higher level

(Fig. 6c). IL-6 promotes the growth and differentiation of B-cells into plasma cells. Waag et al. (1990) have shown with bioassay that pre-exposure of mice to WC-I or CMR optimized the in vitro release of TNF from splenocytes. We could not detect TNF-α by the ELISA method. That discrepancy may reflect differences in sensitivities and target antigens of the assays.

CMR stimulated the production of IL-2 which supports proliferation and differentiation of T- and B-lymphocytes, increases the activity of NK cells, and stimulates the production of IFN-γ, TNF-α and IL-1α. IL-2 was detected only in the supernatants of cultured spleen cells pooled from untreated mice which were stimulated in vitro with CMR (Fig. 6b). No significant levels of IL-2, could be detected in the spleen cell culture supernatants of RVF virus-infected CMR-treated or RVF virus-infected untreated mice, as compared to the control mice. The inability to induce IL-2 in cultured spleen cells pooled from in vivo primed mice even with an in vitro signal in RVF virus-infected or uninfected mice, may be due to prostaglandin E_2 (PGE_2)-induced inhibition; CMR has been shown to stimulate production of PGE_2 (Koster et al., 1985), while PGE_2, in turn, inhibited interleukin-2 production in mice (Walker et al., 1983).

Analysis of lymphocyte subpopulations with respective surface markers showed (Table 2), that 120 h after RVF virus infection, but without CMR administration, the total B-, T_h/T_s, all Ia-positive, and Ia-positive T-cells increased considerably. Administration of 100 μg CMR 24 h prior to RVF virus had a biphasic modulatory effect: by 72 h postinfection total T, T_h/T_s, and Ia-positive T-cells were increased, mimicking the host's immune response to the infectious agent, but, in accordance with the enhanced cytokine responses revealed in this study, in the presence of CMR the immune response to the viral antigen occurred 48 h earlier. At 120 h, the modulatory effect of the CMR was clearly in the negative direction, both in the naive and in the virus-infected host. The implication, if any, of the late negative modulation which followed an earlier augmenting effect requires further studies.

Although the involvement of other microbicidal systems (i.e., oxygen radicals) in the non-specific antiviral protective activity of the CMR, cannot be excluded, it appears that biological activities modulated by CMR have a major role in the induction of the specific and non-specific antiviral resistance. High levels of GM-CSF and IL-1 were detected after in vivo/in vitro or in vitro exposure to CMR. Dendritic cell activity is highly sensitive to macrophage-derived cytokines (Steinman, 1991) including GM-CSF and IL-1. The presence of these cytokines suggests that CMR stimulates antigen-processing cells. Furthermore, increased expression of Ia MHC class II antigen, induction of IFN-γ and other lymphokines by CMR are the markers of adjuvants which lead to enhanced antigen processing and potentiation of humoral and cellular immune response. Studies to elucidate such activities by abrogation of the antiviral mechanism of action with anti-cytokine serum (similarly to the anti-α/β and γ-serum) are in progress.

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