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MDHM, a Macrophage-Stimulatory Product of *Mycoplasma fermentans*, Leads to In Vitro Interleukin-1 (IL-1), IL-6, Tumor Necrosis Factor, and Prostaglandin Production and Is Pyrogenic in Rabbits

PETER F. MÜHLRADT[1]* AND ULRICH SCHADE[2]

Immunology Research Group, Gesellschaft für Biotechnologische Forschung, Mascheroderweg 1, D-3300 Braunschweig,[1] and Forschungsinstut Borstel, D-2061 Borstel,[2] Germany

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***Mycoplasma fermentans*-derived high-molecular-weight material (MDHM) was originally discovered because of its capacity to generate, through the induction of monokine synthesis, cytolytic T lymphocytes in concanavalin A-stimulated thymocyte cultures. This study shows that MDHM-activated macrophages not only released interleukin-6 (IL-6) but also exhibited increased synthesis of cell-associated IL-1 as well as liberation of tumor necrosis factor and prostaglandin. We determined 6-keto prostaglandin F1α since it is the stable metabolite of the bioactive prostacyclin. MDHM appeared to be as potent as lipopolysaccharide in inducing the synthesis of these mediators. Priming with gamma interferon further increased MDHM-mediated IL-6 release. Since monokines can be pyrogenic, we tested the effects of an intravenous injection of MDHM on rectal temperatures and leukocyte counts in rabbits. At 1 h after a bolus injection of MDHM, leukocyte counts dropped to about 35% of the initial values, reflecting a decrease in both lymphocytes and granulocytes. At 4 to 6 h after injection, granulocyte counts began to increase again, whereas lymphocyte counts remained low. No leukocytosis was noted during this time. The lack of leukocytosis can be explained by the failure of MDHM-stimulated macrophages to release IL-1. The property of MDHM to cause IL-6 release from macrophages and the IL-6 growth dependency of the 7TD1 hybridoma cell line were made use of in a coculture assay system to quantitate the activity of MDHM. With this method and macrophages from C3H/HeJ lipopolysaccharide-nonresponder mice, MDHM activity was found to be equally distributed in the mycoplasma growth medium and the sedimented mycoplasmas after sonication.**

The macrophage/monocyte system reacts to a number of bacterial products by the release of diverse mediators with immunomodulatory or inflammatory properties. Several of these bacterial products, such as lipopolysaccharide (LPS), the endotoxin of gram-negative bacteria (16), bacterial lipoprotein (11), or peptidoglycan and fragments thereof (28), are constitutents of the cell wall. Products from mycoplasmas, devoid of a cell wall proper, can also stimulate macrophages to heightened interleukin-6 (IL-6) (13, 19), tumor necrosis factor (TNF) (21, 23), or colony-stimulating factor (22) synthesis. The biochemical nature of the active principle, with the notable exception of the *Mycoplasma arthritidis* product MAS (3), has not been elucidated yet. Most reports, however, are compatible with the active principle being a membrane component.

We previously described *M. fermentans*-derived high-molecular-weight material (MDHM). MDHM was discovered through its capacity to induce monokine-mediated generation of cytolytic T lymphocytes in concanavalin A-stimulated thymocyte cultures (19). MDHM has no effect on adherent cell-depleted thymocyte cultures, nor does it have any noticeable effect on T or B lymphocytes. MDHM acts primarily on macrophages, including those from LPS-nonresponder mice (16a, 19). This study shows that MDHM activated macrophages not only to release IL-6, as previously shown (19), but also to synthesize high levels of IL-1 and moderate levels of TNF as well as to liberate prostaglandin (PG). The in vivo application of MDHM to rabbits resulted in transient leukopenia and an increase in rectal temperature.

MATERIALS AND METHODS

Cultivation of *M. fermentans*. *M. fermentans* D15-86 was grown in GBF-1 medium at 37°C in an atmosphere containing 5% CO_2. GBF-1 medium consists of RPMI 1640–10% fetal calf serum (FCS)–10% (vol/vol) cell sonicate. To prepare the sonicate, we suspended P815 mastocytoma cells at 10^7/ml in phosphate-buffered saline (PBS) and sonicated them in an ice bath by two 2-min bursts in a Branson Sonifier equipped with a conical tip. Cell debris was removed by 45 min of centrifugation at 21,000 × *g*, and the nonsedimented sonicate was passed through a 0.22-μm-pore-size sterile filter. GBF-1 medium was developed to fulfill the need for a medium which was nontoxic for cultured mammalian cells and contained <10 pg of endotoxin per ml, as determined by the *Limulus* assay.

Mycoplasma growth assay with MTT. Growth of *M. fermentans* was monitored by measurement of tetrazolium reduction, an assay developed to determine the inoculum size of *M. pneumoniae* (5). The original protocol was changed as follows. Mycoplasmas from 5 ml of culture fluid were sedimented for 20 min at 12,000 × *g*. Mycoplasmas were taken up in 0.5 ml of GBF-1 medium, transferred in 100-μl portions to the wells of a 96-well flat-bottom microtiter plate, and cultured for 4 h after the addition of 10 μl of a solution of 5 mg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) per ml in PBS. The blue formazan dye was dissolved by the addition of 120 μl of 5%

* Corresponding author.

formic acid in 2-propanol and brief treatment in a sonic bath and was then measured at 577 nm in an enzyme-linked immunosorbent assay (ELISA) reader (MR580; Dynatech Laboratories Inc., Alexandria, Va.).

Preparation of MDHM. Three different preparations were used in this study. MDHM-A was prepared from culture media of *M. fermentans*-infected HL60 cells by ammonium sulfate precipitation (90% saturation), dialysis of the redissolved precipitate against PBS, removal of insoluble material by centrifugation for 1 h at 21,000 × *g*, and filter sterilization as described previously (19). MDHM-S was medium from 3-day-old cultures of *M. fermentans* grown in GBF-1 medium which had been centrifuged for 45 min at 21,000 × *g* to remove the mycoplasmas. MDHM-M was prepared from *M. fermentans* grown in GBF-1 medium, sedimented for 45 min at 21,000 × *g*, and disrupted by a 30-s burst of sonication (60 W) with a Branson Sonifier equipped with a conical tip. Sonication was performed in RPMI 1640–5% FCS. Alternatively, for injection into rabbits, MDHM-M was prepared from mycoplasma sediment which was washed three times with endotoxin-free PBS (Fresenius AG, Bad Hoburg, Germany), taken up in endotoxin-free PBS, and frozen-thawed three times. Such preparations were adjusted to about 200 μg of mycoplasma protein per ml, as determined by the Lowry method. The preparation contained <10 pg of LPS per ml, as determined by the *Limulus* test. MDHM preparations were kept frozen at −20°C until use.

LPS. LPS was prepared from *Salmonella typhimurium* S form by phenol extraction and ultracentrifugation.

Animals. Female CBA/J and C3H/HeJ mice were purchased from Charles River (Sulzfeld, Germany) and used at the age of 3 to 6 months. Rabbits were between 9 and 13 months old.

Cytokines. Murine recombinant gamma interferon (rIFN-γ) (1×10^4 to 2×10^4 U/μg) was a kind gift from G. R. Adolph, Ernst-Boehringer Institut für Arzneimittel-Forschung, Vienna, Austria. Activity units are those defined by the virus protection assay with mouse L cells and encephalomyelitis virus. Murine recombinant IL-4 (rIL-4) (2×10^4 U/μg) was purchased from Genzyme. Activity units are those defined by the supplier. Human rIL-1 (1.25×10^5 U/μg) was purchased from Genzyme. Activity units are those defined by Günther et al. (10). Human rIL-6 (10^5 U/μg) was obtained from Boehringer, Mannheim, Germany. Activity units are those defined by van Snick et al. (27).

Cell culturing. Mice were killed by CO_2 asphyxiation, and resident peritoneal cells were obtained by washing the peritoneal cavity with chilled RPMI 1640–1% FCS–2 mM glutamine–2.5×10^{-5} M 2-mercaptoethanol. Cells were adjusted to 5×10^5/ml and incubated in 0.6-ml portions in 24-well tissue culture plates for the indicated times (routinely 18 h) at 37°C in a moist atmosphere containing 7.5% CO_2. Nonadherent cells were removed by gentle washing, fresh medium with or without macrophage activator was added, and cultivation was resumed for various times.

Bioassays of cytokines. IL-6 was determined with the hybridoma cell line 7TD1 (27) with rIL-6 as an internal standard (19). IL-1 was determined with the D10 G.4.1 helper T cell line (10) with human rIL-1 as an internal standard. This assay does not detect IL-6 (12). TNF alpha (TNF-α) activity was determined in a cytolytic cell assay with the mouse fibrosarcoma line WEHI 164, clone 13 (kindly supplied by T. Epevik) (9). In brief, WEHI 164 cells (2×10^4) were incubated with serial dilutions of the sample in 96-well microtiter plates. After 18 h MTT (100 μg per well) was added, and the reaction was stopped after 4 h by the addition of 5% formic acid in isopropanol. Reduced MTT was determined in an ELISA reader. One unit of TNF-α per milliliter is defined as the reciprocal dilution causing 50% cell destruction. The TNF-α specificity of the reaction was established in control experiments in which specific antiserum against murine TNF-α was added. It was found to neutralize all cytolytic activity.

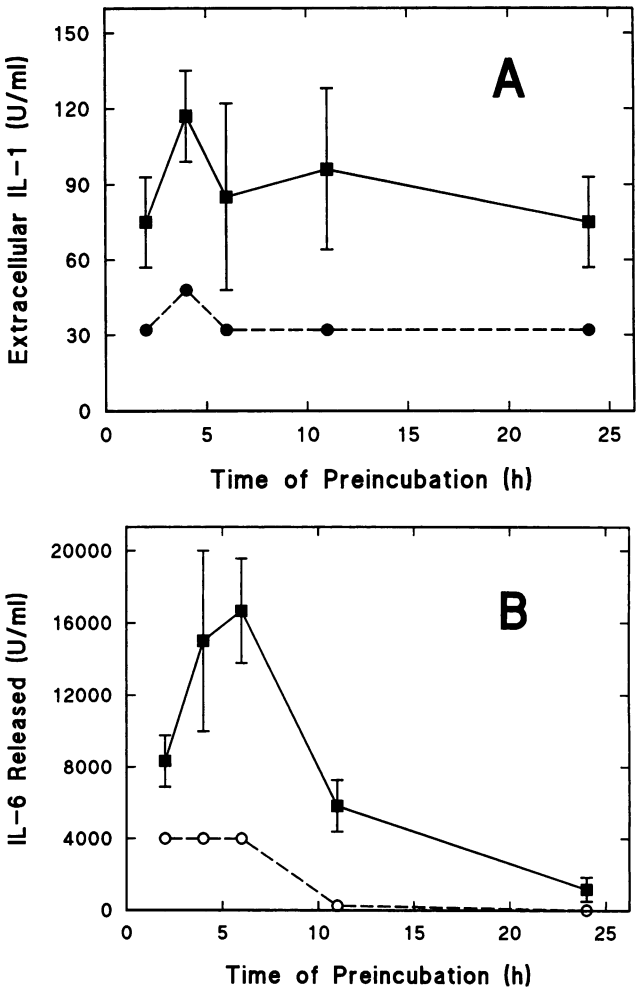

FIG. 1. Extracellular IL-1 and IL-6 in culture media of peritoneal cells after various lengths of preincubation in response to MDHM. Resident peritoneal cells from CBA/J mice were cultured for the indicated times in RPMI 1640–10% FCS. Nonadherent cells were removed by gentle washing, and 0.2% (vol/vol) MDHM-A in RPMI 1640–1% FCS (solid line) or fresh control medium (broken line) was added. Culture medium was harvested after a further 24 h of incubation and assayed for IL-1 with the D10 6.4.1 helper T cell line (A) and for IL-6 with the 7TD1 hybridoma cell line (B). Values shown are means ± standard deviations for triplicate cultures, which were assayed in duplicate.

Estimation of MDHM activity. 1.2×10^3 resident peritoneal cells from C3H/HeJ mice were precultured in 200-μl volumes in RPMI 1640–1% FCS in a 96-well microtiter plate overnight. Cells were washed to remove spontaneously formed IL-6 (see below), and 10^3 7TD1 cells in 50 μl of RPMI 1640–5% FCS and 50 μl of a serial dilution of MDHM in this medium were added. After 3 days of culturing MTT was added, and reduced MTT was measured after 4 h as described above.

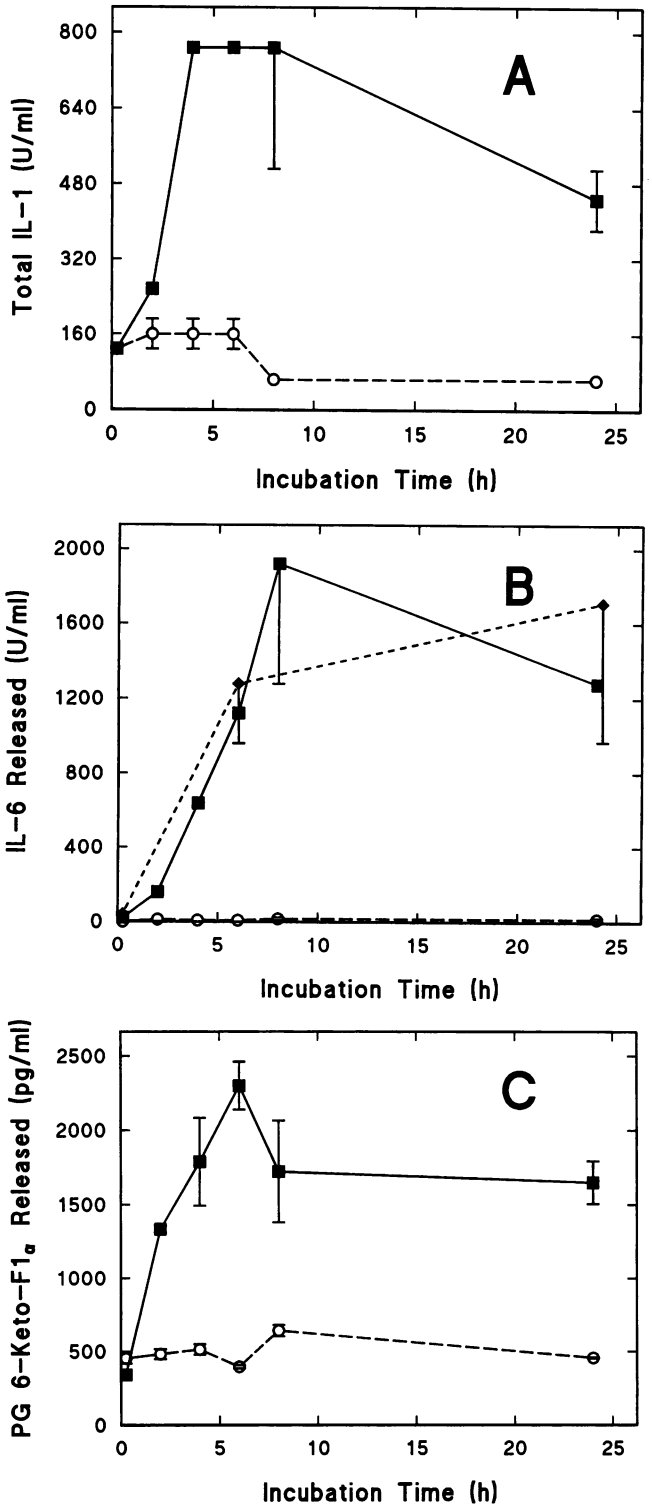

FIG. 2. Kinetics of the synthesis of IL-1, IL-6, and PG in macrophage cultures stimulated with MDHM. Resident peritoneal cells from CBA/J mice were cultured for 19 h in RPMI 1640–1% FCS. Nonadherent cells were removed by gentle washing, and 0.2% (vol/vol) MDHM-A in RPMI 1640–1% FCS or control medium was added. (A) Total IL-1 in cultures that were thawed and frozen three times was assayed after the indicated times with the D10 G.4.1 helper T cell line. Symbols: ■, MDHM stimulated; ○, medium controls. (B) Culture media from parallel cultures were tested for IL-6 with the 7TD1 hybridoma cell line. Symbols: ■, MDHM stimulated; ◆, treated with 0.1 μg of phenol-extracted *S. typhimurium* S-form LPS per ml; ○, medium controls. (C) Culture media from another set of parallel cultures were assayed for 6-keto-$PGF_{1\alpha}$ (PG 6-Keto-$F1_\alpha$) with a radioimmunoassay. Symbols: ■, MDHM stimulated; ○, medium controls. Values shown are means ± standard deviations for triplicate cultures which, for monokine determinations, were assayed in duplicate.

Radioimmunoassay of 6-keto-$PGF_{1\alpha}$. 6-Keto prostaglandin F1α (6-keto $PGF_{1\alpha}$) was determined radioimmunologically as described previously (18).

RESULTS

MDHM-mediated production of inflammatory mediators by peritoneal macrophages. Resident peritoneal macrophages, freshly isolated by adherence to plastic and kept in control medium with 1% FCS, synthesized measurable activities of IL-1 and IL-6, even in the absence of any obvious stimulants. Synthesis of these mediators could, however, be considerably increased by the addition of MDHM-A (Fig. 1A and B). Stimulation by 0.1 μg of LPS per ml resulted in monokine synthesis of a similar extent (data not shown). Spontaneous liberation of mediators became negligible after preculturing of the adherent cells for 15 h, which is why most of our experiments were carried out with precultured cells to which stimulants were added with a simultaneous exchange of medium.

Under these experimental conditions, no IL-1 activity above that in control cultures was found in the culture medium upon stimulation with LPS or MDHM. Only conditions leading to cell damage, e.g., LPS concentrations exceeding 10 μg/ml or cultivation of the macrophages in serum-free medium, resulted in increased extracellular IL-1 (data not shown). Cell-associated IL-1 was therefore routinely determined in cell lysates obtained after three cycles of freezing-thawing of the cultures. IL-6 was found exclusively in the culture medium.

Using the above conditions, we next studied the kinetics of MDHM-mediated synthesis of monokines and PG. At some time points, LPS-stimulated positive control cultures were included for comparison. At 6 and 24 h after stimulation with 0.1 μg of LPS per ml, IL-1 reached 256 and 448 U/ml, respectively. LPS-mediated IL-6 release after these times was as high as MDHM-mediated IL-6 release (Fig. 2B). The data, all taken from the same representative experiment, are presented in Fig. 2. MDHM appeared to be as potent as LPS in inducing the synthesis of IL-1, IL-6, and PG. We determined 6-keto-$PGF_{1\alpha}$ since it is the stable metabolite of the bioactive prostacyclin. Whereas IL-1 and IL-6 titers did not vary appreciably from one experiment to another, TNF activity, when determined at its maximum after 6 h, varied from 250 U/ml in the experiment shown in Fig. 2 to only 75 U/ml in another experiment in which IL-6 release was consistently high.

Modulation of monokine release by PG and IFN-γ. Under our experimental conditions with precultured macrophages, no inhibitory PG concentrations which would interfere with, e.g., IL-6 synthesis, were reached. However, when freshly isolated cells were used at concentrations above 10^5 cells per 2 cm^2, PG release in freshly isolated macrophage cultures reached levels above 4,000 pg/ml and inhibited further monokine synthesis (data not shown).

Because it was reported that IL-4 (6) and gamma interferon (IFN-γ) (17) can modulate the tumoricidal activity of

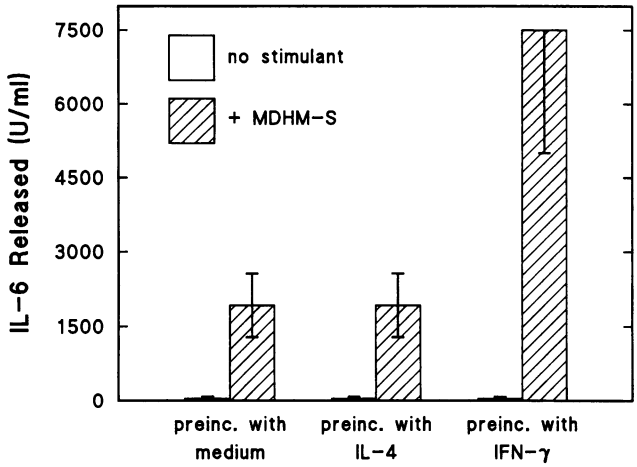

FIG. 3. Effects of preincubation (preinc.) with IFN-γ on MDHM-mediated IL-6 release. Resident peritoneal cells from C3H/HeJ mice were cultured for 24 h in RPMI 1640–1% FCS. Where indicated, 100 U of murine rIL-4 or 50 U of murine rIFN-γ per ml was added. Nonadherent cells and lymphokines were removed by gentle washing, and fresh control medium or 10% (vol/vol) MDHM-S in RPMI 1640–1% FCS was added. Culture medium was harvested 24 h later and assayed for IL-6 with the 7TD1 hybridoma cell line. Values shown are means ± ranges for duplicate cultures, which were assayed in duplicate.

macrophages and, moreover, that IFN-γ can influence monokine synthesis in response to LPS (2, 4, 24), we assayed MDHM-mediated IL-6 release from resident peritoneal macrophages pretreated for 24 h with 100 U of rIL-4 or 50 U of murine rIFN-γ per ml. The experiment was performed with macrophages from CBA/J and C3H/HeJ LPS-nonresponder mice. In both cases, IFN-γ increased MDHM-mediated IL-6 release. The data obtained with the C3H/HeJ macrophages are shown in Fig. 3.

Effects of MDHM-M on rectal temperatures and leukocyte counts in rabbits. Since MDHM stimulated the in vitro release of IL-6 and TNF, both reportedly mediators of a pyrogenic response (7, 26), we tested the effects of intravenous injection of MDHM-M on rectal temperatures and leukocyte counts in rabbits. Pretrials had indicated that doses above 10 μg/kg were effective. The pyrogenic effects of intravenous injection of 30 μg/kg in three rabbits are shown in Fig. 4. For two of these animals leukocytes were counted and differential blood counts were made before and at various times after the injection of MDHM-M. At 1 h after the application of MDHM-M, the leukocyte counts dropped to 28 and 40%, respectively, of the initial values, reflecting a decrease in both lymphocytes and granulocytes. At 4 to 6 h after injection, granulocyte counts began to increase again, whereas lymphocyte counts remained low. No leukocytosis was noted during this time.

Quantitation of MDHM activity in cultures of *M. fermentans*. The property of MDHM to cause IL-6 release from macrophages and the IL-6 growth dependency of the 7TD1 hybridoma cell line were made use of in a coculture assay system to quantitate the activity of macrophage activators like MDHM. Murine resident peritoneal macrophages were cocultured with 7TD1 cells in the presence of various dilutions of MDHM, and the MDHM-dependent growth of 7TD1 cells was measured by MTT reduction. Such an experiment, with peritoneal cells from C3H/HeJ LPS-nonresponder mice and MDHM-S, is shown in Fig. 5. The activity showing half saturation in this growth curve was defined as 1 U. In this example, 0.3% (vol/vol) MDHM-S corresponds to 1 U; i.e., the sample contained 333 U/ml. With this method, the MDHM activity in a 3-day-old culture of *M. fermentans* in 120 ml of GBF-1 medium was determined in the medium and in the mycoplasmas after sedimentation and sonication; 40,000 U of activity was found in the medium (MDHM-S), and 30,000 U was released by sonication of the mycoplasmas (MDHM-M). Determination of MDHM in the medium at different times during cultivation revealed a time-dependent increase in activity, with a maximum at day 3, the time of maximal metabolic activity of the mycoplas-

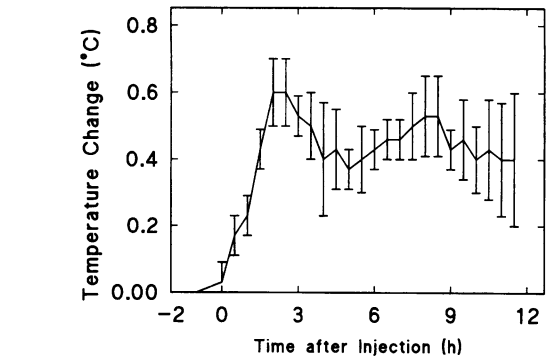

FIG. 4. Febrile responses in rabbits following intravenous injection of MDHM-M. Three rabbits weighing between 4.8 and 5.0 kg were kept in restrainers in a temperature-controlled room at 25°C for 3 h and were then each injected in the ear vein with 160 μg of MDHM-M in pyrogen-free saline. Values given are the mean changes in rectal temperatures ± standard deviations after injection at 0 h.

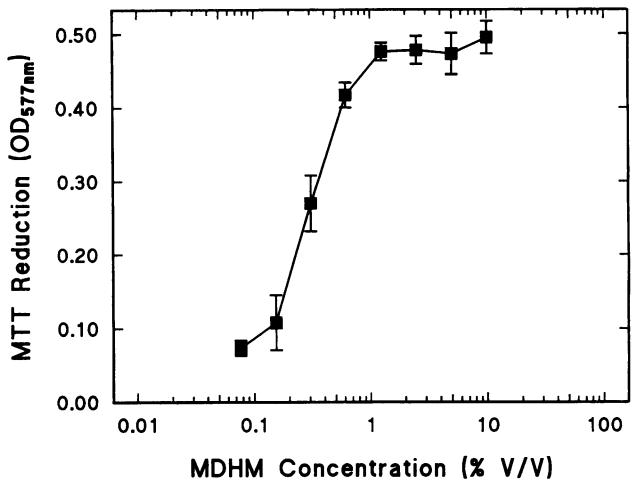

FIG. 5. Quantitation of MDHM activity by coculturing of macrophages with the 7TD1 IL-6 indicator cell line. Resident peritoneal cells (1,200) were cultured in 200-μl volumes in RPMI 1640–1% FCS in a 96-well microtiter plate overnight. Medium was removed, cells were washed, and 1,000 7TD1 cells in 50 μl of RPMI 1640–5% FCS and 50 μl of a serial dilution of MDHM-S in this medium were added. After 3 days of culturing, MTT reduction was measured at 577 nm in an ELISA reader (OD_{577nm}, optical density at 577 nm). Values shown are means ± standard deviations for triplicate cultures.

mas; activity declined to about half on day 7, when few viable mycoplasmas remained.

DISCUSSION

MDHM stimulated the synthesis of several inflammatory mediators in cultured macrophages. Whereas IL-6, TNF, and PG were detected in the culture medium, IL-1 remained cell associated and could only be measured after disruption of the cells.

There exists a complicated regulatory network of inflammatory mediators resulting in the up-regulation of PG (8) and IL-6 (4, 20) by IL-1 and the down-regulation of TNF (1, 20) and IL-1 (20) by IL-6. We did a time course analysis of the formation of these mediators in response to MDHM. The early synthesis of IL-1, reaching a plateau after 4 h and preceding that of IL-6 (Fig. 2), might be interpreted as a stimulus of IL-1 leading to ensuing IL-6 or TNF release. We think this unlikely, as IL-1 remained cell associated and the macrophages were not plated densely enough to allow stimulation by cell-to-cell contact. Conversely, the down-regulation of IL-1 synthesis by IL-6 (20) or PG (15) in the culture medium is compatible with our findings but is not directly supported by them. We did, however, observe a decrease in IL-6 formation when fresh macrophages were seeded at high densities and the PG concentration reached levels above 4,000 pg/ml. This hypothetical down-regulation of IL-6 by PG could be avoided by appropriate culture conditions. Our kinetic data are best compatible with the assumption that MDHM directly causes the synthesis of all these mediators, rather than allowing the synthesis of one monokine, which secondarily stimulates the formation of others.

The response of macrophages and monocytes to stimulation is further influenced by the history of these cells before activation. As has been previously reported, IFN-γ primes monocytes for enhanced LPS-mediated monokine production (2, 4, 24). In agreement with this report, IFN-γ-primed macrophages produced higher IL-6 activities in response to MDHM than did control cells (Fig. 3).

Of the mediators released after in vitro MDHM stimulation, TNF (7) and IL-6 (26) cause fever and, in the case of IL-6, leukopenia as well (26). It was therefore likely that the in vivo application of MDHM would result in similar effects through the in vivo release of these mediators. We indeed observed moderate increases in rectal temperature and leukopenia upon the application of MDHM (Fig. 4). Unlike LPS, which causes leukopenia followed by leukocytosis 4 h after injection (14), MDHM did not lead to leukocytosis. With due caution in the extrapolation of in vivo data from cell culture studies, this phenomenon can be explained by the failure of MDHM-stimulated macrophages to release IL-1. In contrast, LPS leads to a considerable release of IL-1, at least from monocytes (20), and IL-1 can by itself cause leukocytosis (25).

The biochemical nature of MDHM is still largely unresolved. Our earlier data (19) as well as the fact that washed, sonicated mycoplasmas contained active MDHM are compatible with MDHM being membrane associated. The development of an LPS-free medium for *M. fermentans* and the establishment of a semiquantitative test for MDHM activity should be helpful in obtaining more precise information on the structure of MDHM.

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