

Purification and Partial Biochemical Characterization of a *Mycoplasma fermentans*-Derived Substance That Activates Macrophages To Release Nitric Oxide, Tumor Necrosis Factor, and Interleukin-6

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Mycoplasmal products may exert a number of diverse *in vitro* effects on cells of the immune system. A macrophage-activating substance from *Mycoplasma fermentans* was described in this laboratory and named mycoplasma-derived high-molecular-weight material (MDHM). Using synthesis of nitric oxide by peritoneal cells from endotoxin low-responder mice as an assay system, MDHM was purified as follows. After freeze-thawing of *M. fermentans*, MDHM activity was sedimented with the membrane fraction. Membranes were delipidated with chloroform-methanol, and MDHM activity was extracted with octyl glucoside. Coextracted proteins were degraded by proteinase K. MDHM was further purified by reversed-phase high-pressure liquid chromatography and eluted in one major and one minor peak of activity. Neither carbohydrates nor amino acids were found as constituents. MDHM had the following properties: it partitioned into the phenol phase upon phenol-water extraction and into the Triton phase after extraction with Triton X-114. MDHM was not inactivated by either phospholipase A₂ or triglyceride lipases. However, mild periodate treatment led to a >95% loss of activity. Also, alkaline hydrolysis at 25°C completely abolished MDHM activity with a half-life of 2 min. MDHM activity was spread out over a wide molecular weight range upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis of membranes, whereas after proteinase treatment MDHM activity migrated close to the front. These features of MDHM, taken together, speak in favor of an amphiphilic molecule with a lipid moiety carrying fatty acids in ester linkage and a polyol moiety of unknown character. MDHM was active in the nanogram-per-milliliter range, activating macrophages to release nitric oxide, interleukin-6, and tumor necrosis factor.

Mycoplasmas and their products may exert a number of diverse *in vitro* effects on cells of the immune system (reviewed by Ruuth and Praz [22]). It is often difficult to determine the exact causes and mechanisms for such effects because of the complexity of immune reactions involving different interacting cells on the one hand and molecular heterogeneity or poor characterization of the mycoplasma-derived materials on the other.

In previous work we have described a mycoplasma-derived macrophage-activating substance which we named mycoplasma-derived high-molecular-weight material (MDHM). MDHM activity was originally discovered because of its property of stimulating maturation of cytolytic T-cell precursors to cytolytic effector T cells in thymocyte cultures (20). This system was subsequently analyzed in detail, and the primary effect of MDHM was found to be an activation of the few macrophages in the cultures which leads to release of interleukin-1 (IL-1) and IL-6 (18). MDHM was also active on human monocytes (20). Further studies on MDHM were based on liberation of cytokines and arachidonate metabolites from murine peritoneal exudate cells (PECs) as a source of resident macrophages (19). Biochemical characterization of MDHM with such test systems was of necessity slow. We therefore adopted an assay of macrophage activation which was better suited for testing

many samples, i.e., the liberation of nitric oxide (NO) from gamma interferon (IFN- γ)-treated PECs from endotoxin low-responder mice (21). MDHM was shown to be active in this system at very high dilutions, inducing at the same time cytotoxic activity against tumor necrosis factor (TNF)-resistant P815 tumor cells (21).

This paper describes the enrichment and final high-pressure liquid chromatography (HPLC) purification of MDHM, using NO liberation as an assay system. MDHM was shown to be a lipid-like substance capable of activating macrophages at nanogram-per-milliliter concentrations to release NO, IL-6, and TNF. Our findings may help to explain effects of mycoplasma infections in animals and humans on the basis of macrophage activation through MDHM-like substances.

MATERIALS AND METHODS

Reagents and enzymes. Sodium taurocholate, *n*-octyl- β -D-glucopyranoside (octyl glucoside), Triton X-114, and phospholipase A₂ from bee venom were purchased from Sigma, Deisenhofen, Federal Republic of Germany; sodium deoxycholate, *N*-cetylpyridinium chloride, and proteinase K were from Merck, Darmstadt, Federal Republic of Germany; Nonidet P-40 (Triton N-101) was from Fluka, Buchs, Switzerland; 3-[(3-cholaminopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and papain were from Serva, Heidelberg, Federal Republic of Germany; *N*-heptylcarbonyl-methyl α -D-glucopyranoside (HECAMEG) was from Calbiochem, Bad Soden, Federal Republic of Germany; pepsin was from Boehringer, Mannheim, Federal Republic of Germany. The carrier-

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bound triglyceride lipases from *Mucor miki*, *Staphylococcus carnosus*, and *Pseudomonas fluorescens* were a generous gift of H. Erdmann, this institute. Recombinant murine IFN- γ was generously supplied by G. R. Adolf, Ernst Boehringer Institut für Arzneimittelforschung, Vienna, Austria.

Cultivation of *Mycoplasma fermentans*. Our stock *M. fermentans*, strain D15-86, is kept in the infected HL60 cell line from which it was originally isolated (20). A mycoplasma inoculum was prepared by culturing freshly transferred HL60 cells for 3 to 5 days in RPMI 1640–10% fetal calf serum (FCS), after which time HL60 cells were removed by a 10-min centrifugation step at $400 \times g$. Mycoplasmas in the supernatant were then sedimented by a 15-min centrifugation at $12,000 \times g$ and were washed twice in GBF-2 medium, consisting of MEM alpha medium–10% FCS–0.5% (wt/vol) Bacto Tryptone. The medium was filtered through MediaKap-5 Plus hollow-fiber medium filters (0.2- μ m pore size) (Microgon, Laguna, Calif.) before use. Mycoplasmas were inoculated into this medium by fivefold dilution with respect to the original volume of the HL60 culture. Viable mycoplasmas reached a maximum after 3 to 4 days, as judged by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay (12), at which time the mycoplasmas were harvested and washed in endotoxin-free saline (Fresenius, Homburg, Federal Republic of Germany). Protein was then determined according to the procedure of Lowry et al. (15), and the mycoplasmas were stored at -20°C until further use. As a precaution to exclude contamination by other mycoplasmas or a mutational change of strain D15-86, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses of each batch were routinely performed.

Partitioning of MDHM between water–Triton X-114 and water-phenol. Triton X-114 extraction was done on a small scale according to a published procedure adapted for mycoplasmas (36). Briefly, 1 ml of *M. fermentans* suspension corresponding to 0.5 mg of protein was mixed with 20 μ l of Triton X-114 and extracted at 4°C overnight. Upon warming to 37°C , two phases formed; these were separated by brief centrifugation at $11,000 \times g$, removing at the same time some insoluble material. The water and Triton X-114 phases were each reextracted with a fresh water-saturated Triton X-114 phase or Triton X-114-saturated water phase, respectively. Corresponding phases were combined, and protein was precipitated by addition of 5 ml of cold acetone. The precipitated proteins were taken up in 50 μ l of water.

Small-scale extraction with hot phenol was carried out according to the method of Westphal and Jann (35). *M. fermentans*, amounting to 1 mg of protein, was suspended in 2 ml of phenol-saturated water. Two milliliters of water-saturated phenol was added, and the mixture was heated for 10 min in a water bath at 68°C , whereupon the phases merged. The two phases separated again after cooling and brief centrifugation. Each phase was separately collected and dialyzed against several changes of water and finally against 50 mM octyl glucoside in phosphate-buffered saline (PBS).

Enrichment of MDHM. *M. fermentans* from a 4-liter culture, equivalent to about 40 to 50 mg of protein, was subjected to five cycles of freezing and thawing in 45 ml of endotoxin-free saline. The resulting suspension was centrifuged for 1 h at $48,000 \times g$. The sediment was taken up in 10 ml of PBS and extracted twice with 10 ml of chloroform-methanol (2:1, vol/vol) at room temperature. The delipidated water phase was freed of the bulk of organic solvents in vacuo at 37°C and then freeze-dried. The lyophilized material was sonicated with 26 ml of 50 mM octyl glucoside in PBS and treated for 6 min in a boiling water bath. Insoluble material was removed by a

30-min centrifugation step at $27,000 \times g$. The resulting supernatant solution was cleared by filtration through a 0.45- μ m-pore-size filter and pressure concentrated on a YM10 membrane (Amicon, Danvers, Mass.). Salt and detergent were removed by repeated dilutions of the concentrate with pyrogen-free water and subsequent pressure dialysis in the cold. The dialysate was then adjusted to 4 to 5 ml of 0.1 mM Ca^{2+} lactate–7 mM Tris Cl (pH 8.8) and treated for 1 h at 37°C with 5 μ g of proteinase K (Merck) per ml. The reaction was stopped by heating the mixture for 5 min at 100°C , and peptides were removed by extensive dialysis against water in the cold. The inner dialysate was freeze-dried.

Gel electrophoresis. Discontinuous SDS-PAGE was carried out under reducing conditions at pH 8.3 according to the method of Laemmli (13), using 5% acrylamide as a stacking gel and 15% acrylamide as a running gel. Staining was with silver reagent as described previously (30), omitting the periodate oxidation step, which did not result in improved staining. In some cases lanes were sectioned into 4-mm-wide strips, from which MDHM was subsequently extracted with 0.5-ml portions of 50 mM octyl glucoside in PBS at 100°C for 15 min.

HPLC separation of MDHM. MDHM was applied in 1 ml of 25 mM octyl glucoside in PBS to a Micropak MCH 10, 10- μ m RP18 column (Varian; 4 by 300 mm) and fractionated with the following program: time zero, 5% *N,N'*-dimethylformamide, 95% water; at 15 min, 5% *N,N'*-dimethylformamide, 95% water; at 100 min, 5% *N,N'*-dimethylformamide, 95% 2-propanol. The flow rate was 0.6 ml/min, 1.7 min per fraction.

Endotoxin assay. Endotoxin was estimated by the limulus amoebocyte lysate assay with the COATEST endotoxin kit from Chromogenix AB, Mölndal, Sweden.

Mice. Female C3H/HeJ mice were purchased from Bomholtgaard, Ry, Denmark, and used at the age of 10 to 18 weeks.

MDHM activity test by the NO release assay. Previously published tests of NO release by macrophages were based on determination of nitrite as one product of NO decay in a diazo-coupling reaction (6, 17). We modified the system by including nitrate, the second major decay product (16), in the measurement in order to increase the sensitivity of the assay. Resident PECs were used as a source of peritoneal macrophages. Mice were asphyxiated with CO_2 immediately before injection of about 3 ml of ice-cold PBS with 1% FCS into the peritoneal cavity. After gentle massage of the abdomen, PECs were withdrawn, centrifuged in the cold, and adjusted to 2×10^6 cells per ml in Dulbecco's modified Eagle's medium–5% FCS–2 mM glutamine– 2.5×10^{-5} M 2-mercaptoethanol. Recombinant murine IFN- γ (100 U/ml) was added to the PECs, and 50 μ l of cell suspension was added to 50- μ l volumes of a serial 1:2 dilution of MDHM in the above-described medium in 96-well flat-bottom microtiter plates. After 46 h of incubation at 37°C in a humidified atmosphere with 7.5% CO_2 , 10 μ l of a solution containing nitrate reductase from *Aspergillus* species (Boehringer) and NADPH was added to give final concentrations of 20 mU per well and 100 μ M NADPH, respectively. The reaction was stopped after 10 min, and total nitrite was determined by addition of 100 μ l of Griess reagent. Griess reagent consists of a freshly made 1:1 mixture of 0.1% naphthylethylenediamine–2HCl in water and 1% sulfanilamide in 5% H_3PO_4 . The A_{550} was read in an enzyme-linked immunosorbent assay (ELISA) reader. An active MDHM preparation was always included as an internal standard. Units of MDHM activity were defined by the dilution yielding half-maximal NO generation over that in control cultures with IFN- γ but without MDHM (see Fig. 1).

Determination of TNF and IL-6 from PECs. PECs were seeded into 96-well flat-bottom microtiterplates at 6×10^4 cells

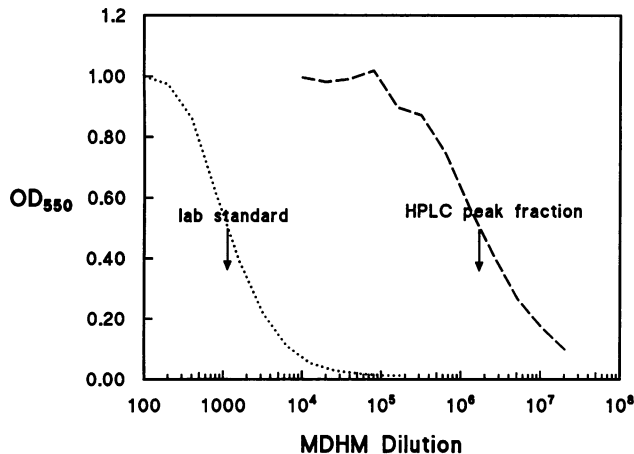


FIG. 1. Definition of activity units for MDHM in the NO release test. PECs (5×10^5) and IFN- γ (50-U/ml final concentration) were added to a serial dilution of MDHM in 96-well microtiter plates. The sum of nitrite and nitrate formed was determined after 48 h. One optical density at 550 nm (OD_{550}) unit corresponds to 12 nmol of NO formed per culture. One unit of MDHM per ml is defined by the dilution yielding half-maximal NO release, the laboratory standard and a sample from an HPLC purification showing activities of 1.0×10^3 and 1.6×10^6 U/ml, respectively.

per well. After 24 h, nonadherent cells were removed from adherent macrophages by shaking on a Wellmixx 4 shaker (Denley Instruments Ltd., Billingshurst, England) for 20 s at setting 8 and removal of the medium. Cultures were washed twice, and 200 μ l of RPMI 1640–5% FCS–2 mM glutamine– 2.5×10^{-5} M 2-mercaptoethanol was added to each well including the appropriate serial dilutions of MDHM. Samples for TNF and IL-6 assays were removed after about 4 and 24 h, respectively.

TNF was determined by a cytotoxicity assay, using L-929 fibroblasts as target cells (1). After exposure to TNF for 16 h, viability was determined with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (Promega) (3) during a 4-h incubation period. One unit of activity is defined as the amount of TNF causing 50% lysis and corresponded to about 4 pg of standard recombinant murine TNF. IL-6 was determined in a capture ELISA, using the IL-6 specific monoclonal antibody from clone MP5-20F3 (Endogen, Boston, Mass.) as a capture antibody and biotinylated monoclonal antibody from clone 6 B4 (33), a kind gift of J. van Snick, for determination of IL-6. Units are defined as in reference 32.

RESULTS

The biological assay system for MDHM. MDHM was previously assayed on the basis of its property of inducing IL-6 from murine macrophages (19). The MDHM-dependent NO generation from IFN- γ -primed peritoneal macrophages turned out to be an equally sensitive assay which was at the same time easier to perform in routine tests (21) (Fig. 1). Units of activity per milliliter were defined as the dilution leading to half-maximal activity in this assay.

Membrane association of MDHM. Pilot experiments had shown that MDHM activity was not extractable by organic solvents such as diethylether, acetone, chloroform-methanol, ethanol, or *n*-butanol (data not shown). In contrast, MDHM was extracted into the phenol phase upon phenol-water extraction according to the method of Westphal and Jann (35) and

TABLE 1. Partition of MDHM between water and triton X-114 or water and phenol

Partition	Fraction (phase)	Vol (μ l)	Protein (mg ml^{-1})	Activity	
				10^3 U ml^{-1}	% of total
Water-Triton X-114	Water ^a	50	0.92	<2	<0.1
	Triton X-114 ^a	50	1.98	500	>99.9
Water-phenol	Water ^b	1,480	0.06	1	0.5
	Phenol ^b	2,920	0.28	96	99.5

^a After acetone precipitation, precipitate taken up in water.

^b After extensive dialysis against 50 mM octyl glucoside in PBS.

into the Triton phase after extraction with Triton X-114 (36) (Table 1). These data suggested that MDHM is to some extent lipophilic and might be a membrane component. Indeed, most of the MDHM activity sedimented with the membrane fraction upon centrifugation when fractured membranes were prepared from *M. fermentans* after repeated freeze-thawing (Table 2).

This finding prompted us to try extractions with each of the detergents sodium deoxycholate, sodium taurocholate, *N*-cetylpyridinium chloride, Nonidet P-40, CHAPS, HECAMEG, and octyl glucoside. Octyl glucoside proved to be optimal on account of its lack of UV absorption, lack of interference with the biological assay, and high critical micellar concentration which allows removal by dialysis.

Attempts at degradation. Earlier experiments had shown that MDHM was not destroyed by nucleases (20). Attempts to destroy MDHM activity by pepsin, papain, and proteinase K were equally unsuccessful. Similarly, MDHM was not inactivated by phospholipase A₂ from bee venom or triglyceride lipases from three different sources. However, mild periodate treatment at pH 5.0 for 1 h at 35°C led to a >95% loss of activity. Also, alkaline hydrolysis at room temperature degraded MDHM activity with a half-life of 2 min (Fig. 2).

Preparation of protein-free MDHM. On the basis of the pilot experiments described above we developed the following purification procedure, which is detailed in Materials and Methods: disruption of *M. fermentans* cells by repeated freeze-thawing, membrane sedimentation and delipidation, extraction of MDHM activity with octyl glucoside, and degradation of coextracted proteins by proteinase K. Biological activity was determined at all separation steps with the NO release assay, and samples were also taken for protein determination as well as for analysis by SDS-PAGE (Fig. 3). Activity was always determined after optimal solubilization in the presence of 25

TABLE 2. Purification of MDHM

Fraction	Vol (ml)	Protein (mg)		Activity (10^6 U)		Sp. act. (10^6 U mg protein ⁻¹)
		ml^{-1}	Total	ml^{-1}	Total	
Membrane suspension	44	1	44	0.32	14	0.3
48,000 \times g supernatant	44	0.11	4.8	0.02	0.9	0.2
48,000 \times g sediment	10	3.4	34	1.28	12.8	0.4
Octyl glucoside extract after delipidation	26	0.18	4.7	0.9	23.4	5.0
YM10 membrane concentrate	20	0.16	3.2	1.28	25.6	8.0
Proteinase K digest, inner dialysate ^a	23.4	0.06	1.4	1.28	30	21.3

^a For the freeze-dried residue (18.3 mg [dry weight]), the total activity was 30×10^6 U and the specific activity was 1.64×10^6 U/mg (dry weight).

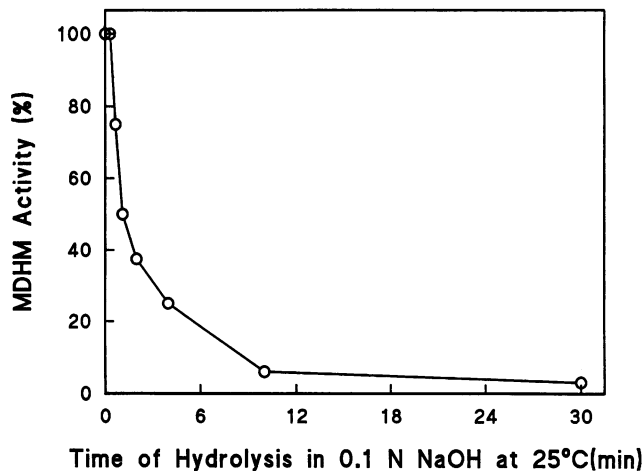


FIG. 2. Alkaline sensitivity of MDHM. A sample containing 1.6×10^5 U of MDHM from an HPLC peak fraction in 2-propanol-water (57:43, vol/vol) was adjusted to pH 13.0 with NaOH. After incubation at 25°C for the indicated times, aliquots were withdrawn, immediately neutralized, and tested for MDHM activity as for Fig. 1.

mM octyl glucoside (see below). A typical example for such an isolation sequence is listed in Table 2. MDHM prepared in this way contained less than 18 pg of endotoxin per ml, as determined by the limulus test, yet was still active at a $>10^6$ -fold dilution.

Activation in the presence of octyl glucoside. When octyl glucoside was removed by dialysis, as, e.g., during the purification, up to 90% of the MDHM activity appeared to be lost. However, short (2- to 3-min) treatment with 25 to 50 mM octyl glucoside in PBS at 100°C fully reconstituted the biological activity. The detergent did not interfere with the biological assay at the high dilutions required to reach half-maximal activity (Fig. 1). The presence of detergent was no longer needed after HPLC purification but still improved the sensitivity of the assay (see Fig. 6).

Behavior after SDS-PAGE. To obtain preliminary information about the apparent molecular weight of MDHM, concen-

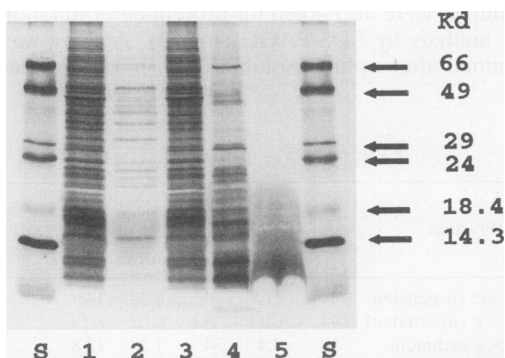


FIG. 3. SDS-PAGE of various fractions from the MDHM purification. Samples were separated on a 15% polyacrylamide gel under reducing conditions. They were applied to the lanes as follows: S, mixture of protein standards; 1, 5 μ g of *M. fermentans* suspension; 2, 1 μ g of 48,000 \times g-centrifuged supernatant; 3, 5 μ g of 48,000 \times g-centrifuged sediment; 4, 5 μ g of YM10 membrane concentrate; 5, same aliquot as in lane 4 after proteinase K treatment and dialysis. The gel was stained with silver reagent.

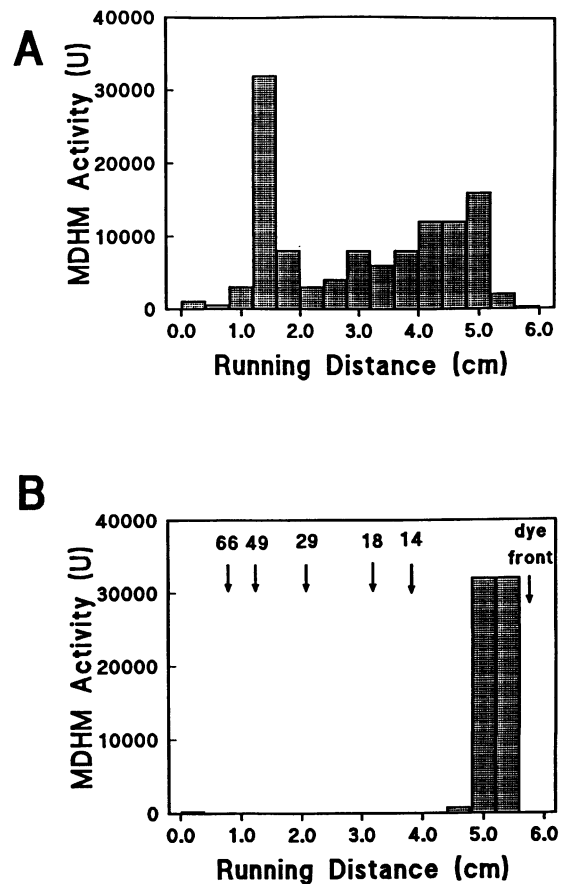


FIG. 4. Determination of MDHM activity after SDS-PAGE. YM10 membrane concentrate (12 μ g) before (A) and after (B) proteinase K digestion was separated by SDS-PAGE as in Fig. 3. The gel was sliced in 4-mm strips immediately after electrophoresis. The strips were extracted with hot octyl glucoside, and the extracts were tested for MDHM activity. Numbered arrows indicate protein sizes (in kilodaltons).

trated octyl glucoside extract of MDHM and a corresponding aliquot of proteinase K-treated extract were subjected to SDS-PAGE. The lanes were sliced immediately after electrophoresis and extracted with octyl glucoside, and MDHM activity was determined in the fractions. As shown in Fig. 4A, there was great heterogeneity of MDHM activity on the gel as long as proteins were still intact. After degradation of the proteins, practically all of the MDHM activity migrated close to the front (Fig. 4B). No particular bands visualized by silver stain appeared to be associated with MDHM activity (compare with Fig. 3).

Purification by HPLC and stability of the final product. MDHM, prepared as described in Table 2, was further purified by reversed-phase HPLC. Initial high losses of activity on the column were overcome by the addition of a small percentage of *N,N'*-dimethylformamide to the solvent gradient. A typical separation is shown in Fig. 5. MDHM activity was eluted in two peaks, a result which was obtained repeatedly in 12 experiments. At this stage of purification MDHM activity could be detected by directly diluting aliquots from HPLC fractions into medium. However, higher activity ensued in both peaks when MDHM was first diluted into 25 mM octyl glucoside and then further diluted into medium. The specific activity of the

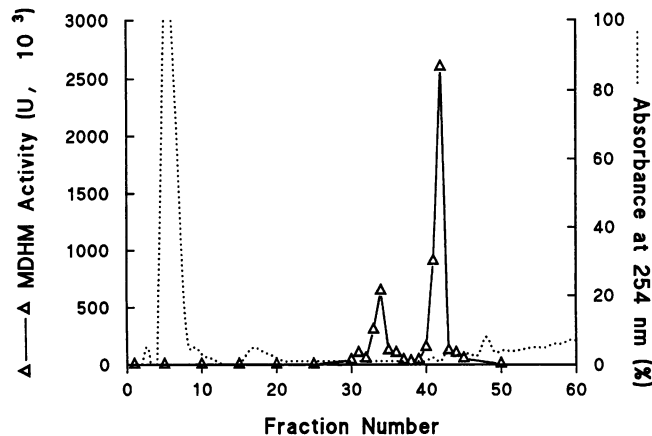


FIG. 5. Reversed-phase HPLC of MDHM. Approximately 3 mg (dry weight) of proteinase K-digested YM10 membrane concentrate was applied to an RP18 column which was eluted with a 2-propanol-water gradient. One-milliliter fractions were collected and tested for MDHM activity.

HPLC-purified main-peak material was 2.5×10^5 U/mg without octyl glucoside and 2×10^6 U/mg in the presence of 25 mM octyl glucoside (see Fig. 6). MDHM from the HPLC column was stored at -20°C and was stable for at least 3 months. When diluted into 25 mM octyl glucoside, no loss of activity was noticeable for at least 4 weeks at 4°C .

Concentration dependency of macrophage activation by MDHM. Determination of dry weight from combined HPLC peak fractions and measurement of biological activity showed that MDHM was still half-maximally active in the nanogram-per-milliliter range or below. The exact concentration depended on whether octyl glucoside was used as a carrier or solubilizing agent in the first dilution step. Half-maximal activity of MDHM was comparable in three different activity tests measuring release of NO, TNF, or IL-6 (Fig. 6).

DISCUSSION

Because of a lack of sufficient material for more-detailed analyses, our attempts at characterizing MDHM have not yet resulted in a definite chemical formula for this biologically highly potent material. Our experiments do allow us to exclude a number of likely structures as candidates and to arrive at preliminary conclusions as to the chemical nature of this compound.

Classical macrophage activators are microbial cell wall components such as endotoxin, murein derivatives, and bacterial lipoprotein and its derivatives. Except for lipoprotein, molluscites are unable to synthesize these compounds. Furthermore, we took great care to exclude contaminations with endotoxin. The following data argue against MDHM being a lipoprotein or lipopeptide. (i) Mycoplasmal lipoproteins give sharp bands in SDS-PAGE, yet MDHM activity was widely spread, possibly because of unspecific association with membrane proteins. (ii) MDHM activity was proteinase K resistant. Although it could be argued that activity may still reside in resulting lipopeptides, the specific activity of purified MDHM is several orders of magnitude higher than that of pure, synthetic lipopeptides which act at $50\text{-}\mu\text{g/ml}$ concentrations (9). (iii) No amino acids were detected in purified MDHM.

Certain strains of molluscites are known to contain lipoglycans (25), formerly designated lipopolysaccharides (26), a term

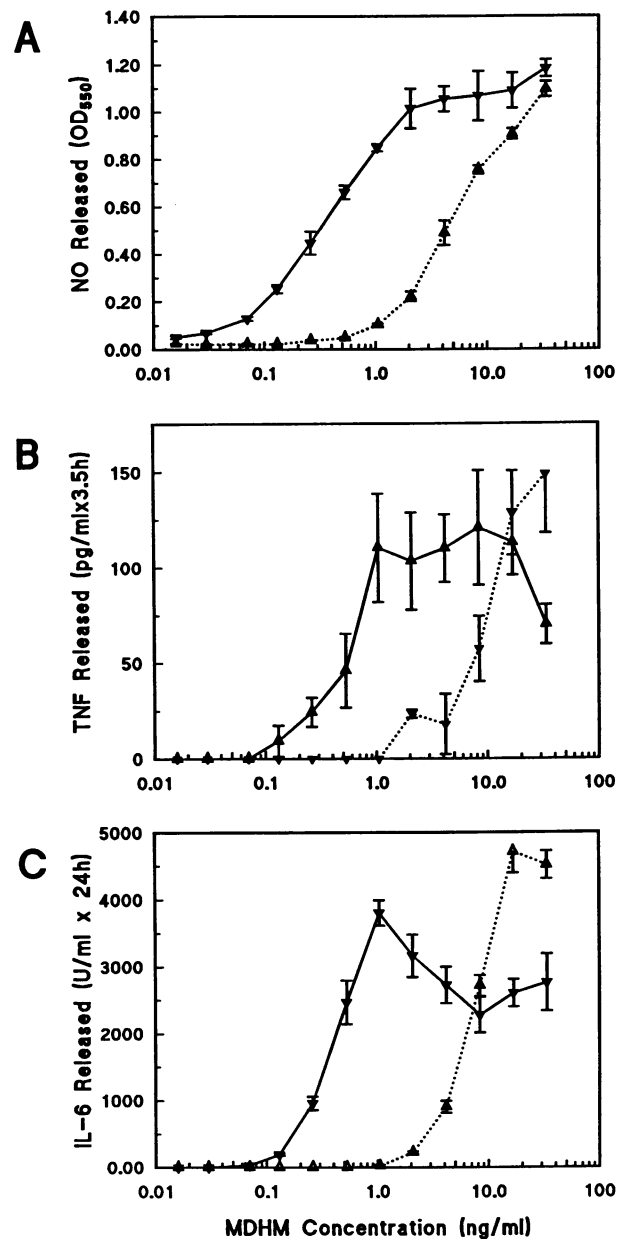


FIG. 6. MDHM concentration dependency of macrophage activation. Tested were releases of NO (A), TNF (B), and IL-6 (C) from PECs (A) or macrophages (B and C). MDHM was pooled from HPLC main-peak fractions. Solid line, MDHM taken up in 25 mM octyl glucoside before dilution with medium; dotted line, MDHM diluted into medium. Data are means \pm standard deviations from triplicate cultures. OD₅₅₀, optical density at 550 nm.

now reserved for lipopolysaccharides derived from gram-negative bacteria. Lipoglycans tend to partition into the water phase upon phenol-water extraction (26). Our data which show that MDHM partitions into the phenol phase argue against MDHM belonging to this group. More convincing are our findings that no carbohydrates could be detected in the peak MDHM fractions from the HPLC, except for traces of glucose which were also found in the neighboring, inactive fractions (17a). Earlier papers have described lipoglycan from *Mycoplasma neurolyticum* as being mitogenically active at $50\text{ }\mu\text{g/ml}$

in rat splenocyte cultures (11) or lipoglycans from *Acholeplasma axanthum* and *Acholeplasma granularum* as showing endotoxin-like properties, including activity in the limulus system (23). However, a contradictory report, also on lipoglycans from *A. axanthum* and *A. granularum*, claimed that these compounds were 10^4 to 10^5 times less active than lipopolysaccharides from gram-negative bacteria in the limulus assay (34). In an independent assay, measuring macrophage-mediated tumor cell killing, these lipoglycans were active at about 1 $\mu\text{g/ml}$ (34).

Our preparation of MDHM was practically inactive in the limulus test, even if undiluted, yet in the macrophage activating assays it was still active at up to a 10^6 -fold dilution. As further shown, purified MDHM was at least 10^2 times more active on a weight-per-milliliter basis in a macrophage-activating system than has been claimed for lipoglycans. It is thus reasonable to suspect that small contaminations of MDHM-like substances in lipoglycan preparations could have seriously interfered with some of the assays performed.

Several macrophage-activating products from various strains of mollicutes have been previously described by different groups (5, 7, 24, 27–29, 31). In most studies crude preparations, such as heat-inactivated mollicutes, culture supernatant (5), or membranes (24), were used, so that comparison of the described activities with MDHM is difficult. Takema et al. have done a preliminary characterization of a macrophage activator from *Mycoplasma gallisepticum* (29). Their data suggest that their material and MDHM have a number of common properties and may be quite similar molecules.

The following features of MDHM speak in favor of an amphiphilic, lipid-like compound: (i) association with the membrane, (ii) enhanced activity in the presence of detergent, particularly as long as other lipids are still present, and (iii) partition into the Triton X-114 phase or phenol phase on the one hand and insolubility in chloroform-methanol on the other. The lipophilic character is likely to be due to substitution with fatty acids in an ester linkage, as mild-alkali treatment led to abolishment of activity with inactivation kinetics typical of fatty acid ester hydrolysis. The hydrophilic moiety is still unknown, but sensitivity to periodate treatment suggests a polyol constituent. It appears from HPLC separation data that MDHM consists of at least two distinct molecular entities, since it gives two well-separated activity peaks.

In our early description of crude, as we now know, membrane-associated MDHM, the apparent molecular weight was that of membrane vesicles (20). The original designation, mycoplasma-derived "high-molecular-weight" material, is certainly a misnomer. The true molecular weight, according to SDS-PAGE, is lower than 14,000. Since MDHM may be expected to form micelles, molecular weight estimation by gel exclusion chromatography is not feasible. A more exact molecular weight determination must await structure elucidation and mass spectroscopy.

Mycoplasmas have been implicated in rheumatic arthritis (2, 8), fever (4), and AIDS (10, 14). It is not unlikely that MDHM-like substances play a role in some of these syndromes. It will therefore be important to test the in vivo properties of purified MDHM, the in vivo stability of this compound, its pyrogenicity, and its antigenicity. We are presently addressing our attention to these problems.

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REFERENCES

1. Aggarwal, B. B., W. J. Kohr, P. E. Hass, B. Moffat, S. A. Spencer, W. J. Henzel, T. S. Bringman, G. E. Nedwin, D. V. Goeddel, and R. N. Harkins. 1985. Human tumor necrosis factor. Production, purification, and characterization. *J. Biol. Chem.* **260**:2345–2354.
2. Barile, M. F., H. Yoshida, and H. Roth. 1991. Rheumatoid arthritis: new findings on the failure to isolate or detect mycoplasmas by multiple cultivation or serologic procedures and a review of the literature. *Rev. Infect. Dis.* **13**:571–582.
3. Buttke, T. M., J. A. McCubrey, and T. C. Owen. 1993. Use of an aqueous soluble tetrazolium/formazan assay to measure viability and proliferation of lymphokine-dependent cell lines. *J. Immunol. Methods* **157**:233–240.
4. Chagnon, A., G. Auzanneau, X. Moreau, S. Othmani, and J. C. Roche. 1986. Responsabilité de *Mycoplasma pneumoniae* dans une fièvre prolongée sans pneumopathie. *Presse Med.* **15**:2021.
5. Dietz, J. N., and B. C. Cole. 1982. Direct activation of the J774.1 murine macrophage cell line by *Mycoplasma arthritis*. *Infect. Immun.* **37**:811–819.
6. Ding, A. H., C. F. Nathan, and D. J. Stuehr. 1988. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. *J. Immunol.* **141**:2407.
7. Gallily, R., M. Salman, M. Tarshis, and S. Rottem. 1992. *Mycoplasma fermentans* (incognitus strain) induces TNF α and IL-1 production by human monocytes and murine macrophages. *Immunol. Lett.* **34**:27–30.
8. Hakkarainen, K., H. Turunen, A. Miettinen, M. Karpelin, K. Kaitala, and E. Jansson. 1992. Mycoplasmas and arthritis. *Ann. Rheum. Dis.* **51**:1170–1172.
9. Hauschildt, S., B. Wolf, A. Lückhoff, and W. Bessler. 1990. Determination of second messengers and protein kinase C in bone marrow derived macrophages stimulated with a bacterial lipopeptide. *Mol. Immunol.* **27**:473–479.
10. Iqbal, M., H. Chowdhury, Y. Koyanagi, S. Kobayashi, N. Yamamoto, T. Munakata, and S. Arai. 1990. *Mycoplasma* and AIDS. *Lancet* **336**:247–248.
11. Kaplan, P.-J., and J. S. Garvey. 1986. *Mycoplasma neurolyticum* membranes: a T-independent antigen in the rat. *Immunol. Invest.* **15**:35–53.
12. Kirchhoff, H., C. Maass, M. Runge, B. Franz, R. Schmidt, H. Quentmeier, and P. F. Mühlradt. 1992. Tetrazolium [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reduction by mycoplasmas. *Int. J. Syst. Bacteriol.* **42**:506–508.
13. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680–685.
14. Lo, S. C., M. S. Dawson, D. M. Wong, P. B. Newton, M. A. Sonoda, W. F. Engler, R. Y. Wang, J. W. K. Shih, H. Alter, and D. J. Wear. 1989. Identification of *Mycoplasma incognitus* infection in patients with AIDS: an immunohistochemical, in situ hybridization and ultrastructural study. *Am. J. Trop. Med. Hyg.* **41**:601–616.
15. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275.
16. Marletta, M. A., P. S. Yoon, R. Iyengar, C. D. Leaf, and J. S. Wisnok. 1988. Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. *Biochemistry* **27**:8706–8711.
17. Migliorini, P., G. Corradin, and S. Betz Corradin. 1991. Macrophage NO $_2^-$ production as a sensitive and rapid assay for the quantitation of IFN- γ . *J. Immunol. Methods* **139**:107–114.
- 17a. Mühlradt, P. F. Unpublished data.
18. Mühlradt, P. F., H. Quentmeier, and E. Schmitt. 1991. Involvement of interleukin-1 (IL-1), IL-6, IL-2, and IL-4 in generation of cytolytic T cells from thymocytes stimulated by a *Mycoplasma*

- fermentans*-derived product. Infect. Immun. **59**:3962–3968.
19. Mühlradt, P. F., and U. Schade. 1991. 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. Infect. Immun. **59**:3969–3974.
 20. Quentmeier, H., E. Schmitt, H. Kirchoff, W. Grote, and P. F. Mühlradt. 1990. *Mycoplasma fermentans*-derived high-molecular-weight material induces interleukin-6 release in cultures of murine macrophages and human monocytes. Infect. Immun. **58**:1273–1280.
 21. Ruschmeyer, D., H. J. Thude, and P. F. Mühlradt. 1993. MDHM, a macrophage-activating product from *Mycoplasma fermentans*, stimulates murine macrophages to synthesize nitric oxide and become tumoricidal. FEMS Immunol. Med. Microbiol. **7**:223–230.
 22. Ruuth, E., and F. Praz. 1989. Interactions between mycoplasmas and the immune system. Immunol. Rev. **112**:133–160.
 23. Seid, R. C., Jr., P. F. Smith, G. Guevarra, H. D. Hochstein, and M. F. Barile. 1980. Endotoxin-like activities of mycoplasma lipopolysaccharides (lipoglycans). Infect. Immun. **29**:990–994.
 24. Sher, T., A. Yamin, S. Rottem, and R. Gallily. 1990. In vitro induction of tumor necrosis factor α , tumor cytolysis and blast transformation by *Spiroplasma* membranes. J. Natl. Cancer Inst. **82**:1142–1145.
 25. Smith, P. F. 1987. Antigenic character of membrane lipoglycans from mollicutes—a review. Isr. J. Med. Sci. **23**:448–452.
 26. Smith, P. F., T. A. Langworthy, and W. R. Mayberry. 1976. Distribution and composition of lipopolysaccharides from mycoplasmas. J. Bacteriol. **125**:916–922.
 27. Stuart, P. M., G. H. Cassell, and J. G. Woodward. 1989. Induction of class II MHC antigen expression in macrophages by *Mycoplasma* species. J. Immunol. **142**:3392–3399.
 28. Sugama, K., K. Kuwano, M. Furukawa, Y. Himeno, T. Satoh, and S. Arai. 1990. Mycoplasma induce transcription and production of tumor necrosis factor in a monocytic cell line, THP1, by a protein kinase C-independent pathway. Infect. Immun. **58**:3564–3567.
 29. Takema, M., S. Oka, K. Uno, S. Nakamura, H. Arita, K. Tawara, K. Inaba, and S. Muramatsu. 1991. Macrophage-activating factor extracted from mycoplasmas. Cancer Immunol. Immunother. **33**:39–44.
 30. Tsai, C.-M., and C. E. Frasch. 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119**:115–119.
 31. Uno, K., M. Takema, S. Hidaka, R. Tanaka, T. Konishi, T. Kato, S. Nakamura, and S. Muramatsu. 1990. Induction of antitumor activity in macrophages by mycoplasmas in concert with interferon. Cancer Immunol. Immunother. **32**:22–28.
 32. van Snick, J., S. Cayphas, A. Vink, C. Uyttenhove, P. G. Coulie, M. R. Ruber, and R. J. Simpson. 1986. Purification and NH₂-terminal amino acid sequence of a T-cell-derived lymphokine with growth factor activity for B-cell hybridomas. Proc. Natl. Acad. Scie. USA **83**:9679–9683.
 33. Vink, A., P. G. Coulie, P. Wauters, R. P. Nordan, and J. van Snick. 1988. B cell growth and differentiation activity of interleukin-HP1 and related murine plasmacytoma growth factors. Synergy with interleukin 1. Eur. J. Immunol. **18**:607–612.
 34. Weinberg, J. B., P. F. Smith, and I. Kahane. 1980. Bacterial lipopolysaccharides and mycoplasma lipoglycans: a comparison between their abilities to induce macrophage-mediated tumor cell killing and limulus amebocyte lysate clotting. Biochem. Biophys. Res. Commun. **97**:493–499.
 35. Westphal, O., and K. Jann. 1965. Bacterial lipopolysaccharides. Extraction with phenol-water and further applications of the procedure. Methods Carbohydr. Chem. **5**:83–91.
 36. Wise, K. S., and M. F. Kim. 1987. Major membrane surface proteins of *Mycoplasma hyopneumoniae* selectively modified by covalently bound lipid. J. Bacteriol. **169**:5546–5555.