Identification of *S*-(2,3-Dihydroxypropyl)cystein in a Macrophage-Activating Lipopeptide from *Mycoplasma fermentans*†

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ABSTRACT: Mycoplasmas are capable of stimulating monocytes and macrophages to release cytokines, prostaglandins, and nitric oxide. The aim of this study was to characterize the chemical nature of the previously isolated [Mühlradt, P. F., & Frisch, M. (1994) *Infect. Immun. 62*, 3801−3807] macrophage-stimulating material "MDHM" from *Mycoplasma fermentans*. Mycoplasmas were delipidated, and MDHM activity was extracted with octyl glucoside and further purified by reversed-phase HPLC. Macrophage-stimulating activity was monitored by nitric oxide release from peritoneal macrophages from C3H/HeJ endotoxin low responder mice. HPLC-purified MDHM was rechromatographed on an analytic scale RP 18 column before and after proteinase K treatment. Proteinase treatment did not diminish biological activity but shifted MDHM elution toward higher lipophilicity, suggesting that the macrophage-stimulating activity might reside in the lipopeptide moiety of a lipoprotein. Proteinase K-treated MDHM was hydrolyzed, amino groups were dansylated, and the dansylated material was isolated by HPLC. Dansylated *S*-(2,3-dihydroxypropyl)cystein (glycerylcystein thioether), typical for Braun's murein lipoprotein, and Dns-Gly and Dns-Thr were identified by tandem mass spectrometry. These amino acids were isolated from biologically active but not from the neighboring inactive HPLC fractions. IR spectra from proteinase K-treated, HPLC-purified MDHM and those from the synthetic lipopeptide [2,3-bis(palmitoyloxy)-(2-*RS*)-propyl]-*N*-palmitoyl-(*R*)-CysSerSerAsnAla were very similar. The data, taken together, indicate that lipoproteins of a nature previously detected in eubacteria are expressed in *M. fermentans* and that at least one of these lipoproteins and a lipopeptide derived from it constitute the macrophage-activating principle MDHM from these mycoplasmas.

Macrophages and other phagocytes constitute the first line of our natural defense system against bacteria. Phagocytes react to microbial stimuli through specific cell surface receptors which bind to typical bacterial products, e.g. N-formylated peptides, or components of microbial cell walls. Thus, peptidoglycan fragments (Staber et al., 1978; Fidler et al., 1990), lipopolysaccharide (Mizel et al., 1978), or murein lipoprotein (Hoffmann et al., 1988, 1989) stimulates macrophages to release cytokines or become cytocidal. Often, macrophage stimulation was at first not recognized as such but was discovered through indirect effects on the immune system such as adjuvanticity (Chedid et al., 1976) or mitogenic activity of B cells (Melchers et al., 1975). Mycoplasmas, although devoid of a cell wall proper, are potent macrophage stimulators (Loewenstein et al., 1983; Quentmeier et al., 1990; Sher et al., 1990; Takema et al., 1991; Gallily et al., 1992; Kita et al., 1992). Again, macrophage stimulation by mycoplasmas or their products was in some cases originally detected through indirect effects, e.g. on T cell development (Mühlradt et al., 1991), but is now more conveniently determined from the release of cytokines (Quentmeier et al., 1990; Sher et al., 1990; Mühlradt & Schade, 1991), prostaglandins (Mühlradt & Schade, 1991), or, more recently, nitric oxide (Ruschmeier et al., 1993; Mühlradt & Frisch, 1994). The most sensitive assay may be inhibition of interferon (IFN)-γ-dependent formation of the class II major histocompatibility complex (Frisch & Mühlradt, 1996). Interactions of mycoplasmas with immune cells (Ruuth & Praz, 1989) or with phagocytes in particular have been recently reviewed (Marshall et al., 1995).

Early experiments on macrophage activation by mycoplasmas were performed with heat-killed mycoplasmas or membrane preparations. The exact chemical nature of mycoplasma-derived macrophage stimulators has remained elusive, and reports from various laboratories are contradictory, ranging from lipoglycan (Seid et al., 1980) to lipids (Salman et al., 1994), peptide linked to lipid or carbohydrate (Yang et al., 1994), or lipoproteins (Herbelin et al., 1994; Kostyal et al., 1994). We recently reported purification and partial characterization of a macrophage-activating material from *Mycoplasma fermentans* which we named mycoplasma-derived high-molecular weight material (MDHM)[1] (Mühlradt & Frisch, 1994). MDHM was sensitive to treatment with periodate and mild alkali but resistant to nucleases, proteinase, or phospholipase A_2, and HPLC-purified MDHM was active in less than nanograms per milliliter concentrations. Phase separation between phenol/water or Triton X114 partition showed the lipophilic properties of MDHM. However, the material was not soluble in common organic solvents and formed liposomes in aqueous media, and

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[1] Abbreviations: Dns = dansyl, 5-(dimethylamino)naphthalene-1-sulfonyl; Gro-Cys, *S*-(2,3-dihydroxypropyl)cystein; rIFN-γ, recombinant interferon-γ; MDHM, mycoplasma-derived high-molecular weight material; PEC, peritoneal exudate cells.

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introduction of MDHM into the culture medium via octyl glucoside increased biological activity about 10-fold. We argued that as MDHM was an amphiphilic molecule it might be associated with protein but that the protein moiety, if any, was unnecessary for biological activity (Mühlradt & Frisch, 1994).

We describe in this paper that MDHM is derived from a lipoprotein of the kind first characterized by Braun and his co-workers and that was found covalently bound to the murein sacculus of Gram-negative bacteria (Hantke & Braun, 1973). The murein prolipoprotein is posttranslationally modified with acylated glycerol and then specifically cleaved by a signal peptidase II [reviewed by Hayashi and Wu (1990) and by Braun and Wu (1994)]. Although DNA sequencing data revealed the appropriate consensus sequence for the signal peptidase II in several mycoplasma species (Dudler et al., 1988; Cleavinger et al., 1994; Christiansen et al., 1994; Ferris et al., 1995), the expression of this particular type of lipoprotein in mollicutes could only be inferred from incorporation studies with labeled fatty acids and cystein (Nyström et al., 1992; Wise et al., 1993). Incorporation of radiolabeled glycerol into the prolipoprotein, using the methodology described for the *Escherichia coli* system (Chattopadhyay & Wu, 1977), turned out to be unfeasible with *M. fermentans* (our own unpubished results) and *Acholeplasma laidlawii* (Nyström et al., 1992). These findings may indicate that neither *M. fermentans* nor *A. laidlawii* metabolizes external glycerol or that this type of posttranslational protein modification does not exist in these mollicutes. Moreover, the murein lipoprotein is not the only species of bacterial lipoprotein. Other types of lipid-modified proteins exist in mollicutes (Nyström & Wieslander, 1992) and other procaryotes (Issartel et al., 1991) so that the above studies, although strongly suggestive, do not unambiguously prove the existence of the murein type lipoproteins in mollicutes, let alone their role as macrophage activators from these organisms.

We therefore examined MDHM for the presence of *S*-(2,3-dihydroxypropyl)cystein (glycerylcystein thioether = Gro-Cys) typical for Braun's murein lipoprotein (Hantke & Braun, 1973). Gro-Cys was isolated from hydrolysates of HPLC-purified MDHM and identified as the dansylated derivative by mass spectrometry. Ours may be the first direct proof for the presence of this modified amino acid in mycoplasmas. Otherwise, the murein lipoprotein type is very common in bacteria as recently reviewed (Braun & Wu, 1994; Sutcliffe & Russell, 1995).

MATERIALS AND METHODS

Growth of M. fermentans. M. fermentans strain D15-86, originally recovered from a contaminated HL60 culture, was grown at 37 °C in a 7.5% CO_2 atmosphere for 3 d in GBF-3 medium consisting of bicarbonate-buffered MEM alpha medium, 10% heat-inactivated newborn calf serum (Sigma, Deisenhofen, Germany), 0.5% (w/v) Bacto Tryptone, with 5 mM fructose, and 10 mg/L adenosine, guanosine, cytidine, uridine, 2′-deoxyadenosine, 2′-deoxyguanosine, 2′-deoxycytidine, and 2′-deoxythymidine. Mycoplasma cultures were split 1/10 every other day until harvest and washed with pyrogen-free saline.

Isolation of MDHM. MDHM was extracted and purified as described (Mühlradt & Frisch, 1994). Briefly, mycoplasmas were delipidated with chloroform/methanol and extracted with hot *n*-octyl β-D-glucopyranoside. The octyl glucoside extract containing the "native" MDHM activity was dialyzed, treated with proteinase K, again dialyzed, and freeze-dried. In some instances, proteinase K digestion was omitted. Native or proteinase K-treated MDHM was finally purified by reversed-phase HPLC.

HPLC. HPLC was performed on analytical (4 × 250 mm) or preparative (10 × 250 mm) Macherey and Nagel (Düren, Germany) columns with Nucleosil 120-7C18 stationary phase. Native or proteinase K-treated MDHM was solubilized in a minimal volume of 25 mM octyl glucoside in 0.1 M ammonium acetate (pH 7.0) and 0.05 M $CaCl_2$. Insoluble material did not contain MDHM activity and was sedimented. MDHM in the supernatant was applied to the column and was eluted at 40 °C with a water/2-propanol gradient. Dansylated samples were separated at 30 °C with a gradient system similar to that of Weiner and Tishbee (1981) but with the following modifications: solvent A, 50 mM triethylammonium acetate (pH 5.8); solvent B, solvent A/acetonitrile/2-propanol (245/250/5) (v/v/v).

Determination of Total Phosphate. Total inorganic phosphate was determined after oxidative digestion as described (Lowry et al., 1954).

Cell Culture and Macrophage Activation (NO Release Assay). Female C3H/HeJ endotoxin low responder mice were purchased from Bomholtgaard, Ry, Denmark, and used at the age of 10−18 weeks. Animals were asphyxiated with CO_2 immediately before injection into the peritoneal cavity of about 3 mL of ice-cold Hank's balanced salt solution with 2% fetal calf serum. Peritoneal exudate cells (PEC) were withdrawn, centrifuged in the cold, and suspended in DMEM medium containing 5% heat-inactivated FCS, 2 mM glutamine, and 2.5×10^{-5} M 2-mercaptoethanol (culture medium). Murine rIFN-γ at a final concentration of 50 u/mL was added with the PEC at a density of 1×10^5 PEC per well to serial dilutions of MDHM in 96-well flat-bottom plastic culture dishes. HPLC-purified MDHM was dissolved in 25 mM octyl glucoside in PBS and further diluted with the medium. The detergent had no effects on cell cultures at the high dilutions used, octyl glucoside concentrations being maximally 625 μM. DMEM was used as it does not contain nitrate and allows determination of the sum of nitrate and nitrite as the main decay products of nitric oxide. After incubation for 45 h at 37 °C in a humidified atmosphere of 7.5% CO_2, nitrate was reduced with NADPH and nitrate reductase, and nitrite was measured in a diazo-coupling reaction as described (Mühlradt & Frisch, 1994; Stuehr & Marletta, 1985). Units of MDHM activity are defined as the dilution required to reach half-maximal stimulation (Mühlradt & Frisch, 1994).

Isolation of Dansylated Gro-Cys. Samples were dried in a Wheaton vial and taken up in 500 μL of 4 N methanesulfonic acid. The vials were degassed, air was replaced by nitrogen, and the samples were hydrolyzed for 18 h at 110 °C. After neutralization of most of the acid by addition of NaOH, but still at an acidic pH, samples were extracted three times with cyclohexane. The aqueous phase was neutralized with 0.2 mL of 1 M $NaHCO_3$, and 0.7 mL of 10% dansyl chloride in acetone was added. The mixture was stirred at room temperature for several hours in the dark. The mixture was extracted three times with cyclohexane and three times with chloroform. The aqueous phase was then subjected to

HPLC. Authentic *S*-(2,3-dihydroxypropyl)cystein and the lipopeptides [2,3-bis(palmitoyloxy)-(2-*RS*)-propyl]-*N*-palmitoyl-(*R*)-CysSerLys$_4$ and [2,3-bis(palmitoyloxy)-(2-*RS*)-propyl]-*N*-palmitoyl-(*R*)-CysSerSerAsnAla were generously provided by G. Jung and synthesized as described (Metzger et al., 1991).

Fast Atom Bombardment Mass Spectrometry. FAB-MS in the positive and negative mode was performed on the first of two mass spectrometers of a tandem high-resolution instrument of $E_1B_1E_2B_2$ configuration (JMS-HX/HX110A, JEOL, Tokyo) at a 10 kV accelerating voltage. Resolution was set to 1/1000. The JEOL FAB gun was operated at 6 kV with xenon as the reactant gas. Thioglycerol and glycerol were used as matrices in the positive and negative modes, respectively.

Tandem Mass Spectrometry. Positive and negative daughter ion spectra were recorded using all four sectors of the tandem mass spectrometer. High-energy collision-induced dissociation took place in the third field-free region. Helium served as the collision gas at a pressure sufficient to reduce the precursor ion signal to 30% of the original value. The collision cell was operated at ground potential. Resolution of MS2 was set to 1/1000. FAB-MS/MS spectra (linked scans of MS2 at a constant B/E ratio) were recorded at 300 Hz, filtering with a JEOL DA 7000 data system using JEOL Complement software Version 2.41.

IR Spectrometry. Samples were dried over KOH, mixed with KBr, compressed into a disk, and measured in a Nicolet 20DXB FTIR spectrometer.

RESULTS

Comparison of "Native" and Proteinase K-Treated MDHM by Reversed-Phase HPLC. Mycoplasmas from 1.75 L of culture, corresponding to 25.5 mg of mycoplasma protein, were delipidated and extracted with octyl glucoside. The extract was dialyzed and freeze-dried. The resulting 25.5 mg of residue, containing nucleic acid and native MDHM with an activity of ca. 4×10^6 u, was applied to a preparative HPLC column such as in Figure 2. The fractions were tested for macrophage stimulatory activity with the NO release assay. MDHM activity eluted in one major and one minor peak in fractions 34−36 and 44−46, respectively (not shown). Two 1 mL aliquots with MDHM corresponding to ca. 1.5×10^5 u from fraction 35 were dried in vacuo. One portion was treated with 10 μg of proteinase K for 1 h at 37 °C, and the other was kept for the same time in buffer without protease as the control. Both were then subjected to analytical HPLC. As noted previously (Quentmeier et al., 1990), there was no significant destruction of macrophage-stimulating activity by proteinase treatment, but there was a shift in the elution pattern of MDHM activity toward higher lipophilicity (Figure 1).

Preparation of Proteinase K-Treated MDHM, Hydrolysis, and Derivatization of Peak and Neighboring Fractions with Dansyl Chloride. Mycoplasmas from 5.4 L of culture, corresponding to 72 mg of mycoplasma protein, were delipidated and extracted with octyl glucoside. The extract was dialyzed, proteinase K-treated, again dialyzed, and freeze-dried. The resulting 15 mg of residue with an MDHM activity of 6×10^7 u was applied to a preparative HPLC column as shown in Figure 2. MDHM activity was measured with the NO release assay, and MDHM peak

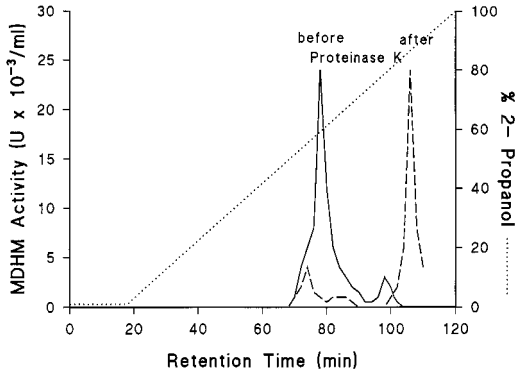

FIGURE 1: Reversed-phase HPLC of MDHM before and after proteinase K treatment. MDHM activity was extracted from delipidated mycoplasmas with octyl glucoside. MDHM activity was then isolated after preparative HPLC, and equal aliquots from peak fractions were treated with proteinase K or control buffer. These samples were then rechromatographed on a 4 × 250 mm RP 18 column. Eluant fractions were tested for macrophage stimulatory activity with the nitric oxide assay. MDHM units are defined by the dilution required to reach half-maximal stimulation.

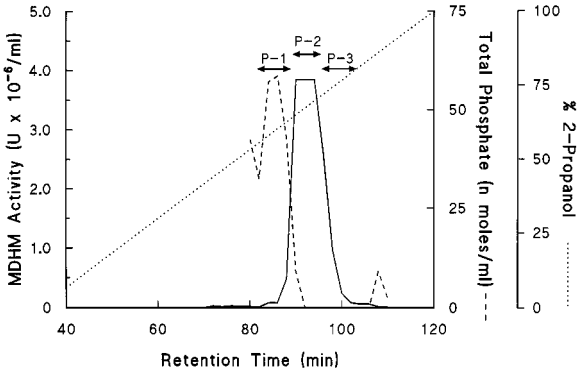

FIGURE 2: Isolation of proteinase K-treated MDHM by HPLC. MDHM activity was extracted from delipidated mycoplasmas with octyl glucoside and the extract pressure dialyzed and digested with proteinase K. The digest was chromatographed on a 10 × 250 mm RP 18 column. Fractions were analyzed for MDHM activity and inorganic phosphate and combined in three pools as indicated.

fractions as well as those before and after this peak were pooled as indicated in Figure 2. Fractions around MDHM activity were also assayed for total inorganic phosphate to monitor remaining phospholipids (nucleic acids and oligonucleotides eluting at retention times of less than 50 min). The pooled material was hydrolyzed, and amino groups were dansylated.

Reversed-Phase HPLC of Dansylated S-(2,3-Dihydroxypropyl)cystein (Dns-Gro-Cys). Dansylated material from pools 1−3 was separated by HPLC, and fractions were collected (Figure 3). Comparison of the elution patterns of these pools monitored by UV absorption at 286 nm showed characteristic peaks eluting after 40 min in the dansylated hydrolysate from pool 2 (see arrows in Figure 3) which were missing in the chromatograms from adjoining pools 1 and 3 (not shown). The peaks contained material with a UV spectrum typical for the dansyl group. The retention times corresponded to those of authentic Dns-Gro-Cys, Dns-Thr, and Dns-Gly respectively. The material was collected and further characterized by mass spectrometry.

Identification of Dns-Gro-Cys by Negative FAB Mass Spectrometry. We chose tandem mass spectrometry (MSMS) (McLafferty, 1983) as ideally suited for analysis of the dansylated material. In the first of the two mass spectrom-

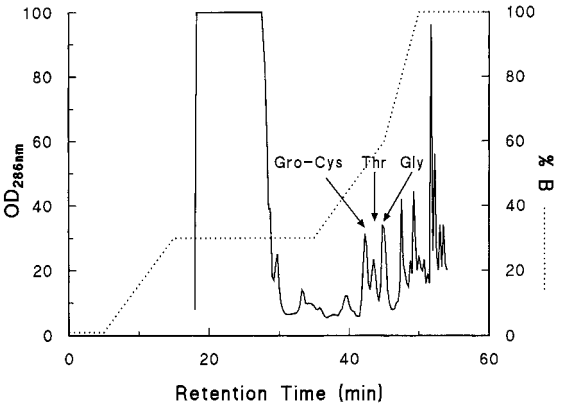

FIGURE 3: HPLC of hydrolyzed, dansylated material from pool 2 (Figure 2). The peaks indicated by arrows were absent from pools 1 or 3 and were identified by tandem mass spectrometry as dansylated Gro-Cys, Thr, and Gly respectively. The large peak eluting after 20 min is due to excess dansyl sulfonic acid.

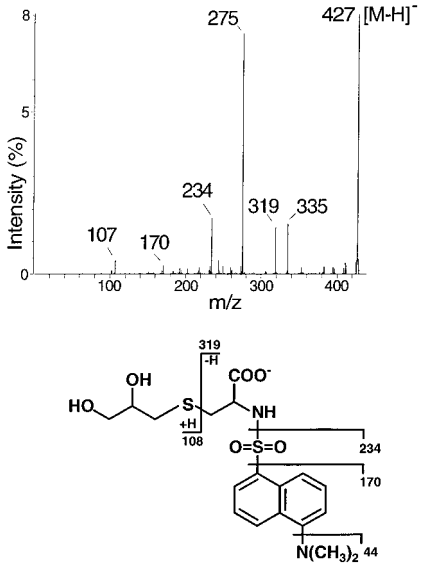

FIGURE 4: Tandem mass spectrum of dansylated *S*-(2,3-dihydroxypropyl)cystein (Gro-Cys). The proposed fragmentation pattern is indicated.

eters, the ion of interest can be selected and then in the following field-free region decomposed by collision with a neutral gas (collision-induced dissociation) (Hayes & Gross, 1990). The resulting fragments are then analyzed in the second mass spectrometer.

The dansylated material eluting as the first peak after 40 min (see Figure 3) was identified as Gro-Cys by comparing MSMS spectra of the hydrolyzed and derivatized biological material with those of an authentic sample of dansyl-Gro-Cys. MSMS was performed in the positive as well as negative ion mode. In the positive FAB mode, major fragment ions for Dns-Gro-Cys at *m/z* 170 and 171 due to the (dimethylamino)naphthalene group were observed (Addeo et al., 1975; Walther et al., 1987) (our data not shown). The dansylated compounds gave stronger quasimolecular signal intensities in the negative ion mode, and this is exemplified by the negative ion FAB-MS of Dns-Gro-Cys shown in Figure 4. Dns-Gro-Cys was observed as the $[M - H]^-$ ion at *m/z* 427, and the MSMS spectrum was identical with that of authentic material. Collision-induced decomposition yielded several characteristic fragments: *m/z* 319 resulting from a loss of thioglycerol due to an intramolecular hydrogen shift and the major fragment (*m/z* 275), probably arising from

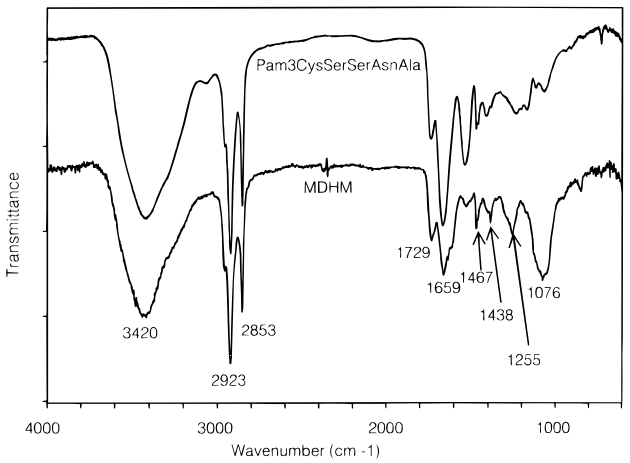

FIGURE 5: Infrared spectra of proteinase K-treated, HPLC-purified MDHM and the synthetic peptide [2,3-bis(palmitoyloxy)-(2-*RS*)-propyl]-*N*-palmitoyl-(*R*)-CysSerSerAsnAla (Pam3SerSerAsnAla). The dried samples were measured in KBr.

the consecutive loss of a dimethylamine radical and thioglycerol. Further fragments at *m/z* 234 and 170 (dansyl- and dimethylnaphthalene, respectively) resulted from α-cleavage. The thioglycerol anion was also observed at *m/z* 107, likewise due to α-cleavage. The ion at *m/z* 335, as well as some minor ones, results from an accompanying low-intensity background matrix ion at *m/z* 427. Dansylated material eluting after the Gro-Cys peak was identified as the dansyl amino acids threonine and glycine, exhibiting strong $[M - H]^-$ ions at *m/z* 351 and 307, respectively. Both compounds gave MSMS spectra with characteristic ions at *m/z* 234, 170, 154, and 64 originating from the dansyl group.

Comparative IR Spectroscopy of Proteinase K-Treated MDHM and Authentic Lipopeptide. Proteinase K-treated MDHM was prepared and isolated by HPLC as above. Four million units from the fraction containing peak activity was subjected to IR spectrometry, and its transmission spectrum was compared with that of the lipopeptide [2,3-bis(palmitoyloxy)-(2-*RS*)-propyl]-*N*-palmitoyl-(*R*)-CysSerSerAsnAla (Figure 5). The IR spectra were very similar, showing intensive C−H stretching bands at 2923 and 2853 cm^{-1} due to the fatty acid alkyl chains and typical ester and amide absorptions at 1729 and 1659 cm^{-1}, respectively. Complete identity of these spectra cannot be expected, as the amino acid and fatty acid composition were not identical. We identified by mass spectrometry C16:0, C18:1, C18:0 fatty acids in the cyclohexane extracts from the hydrolysate from pool 2 material (see Figure 2). They did not result from contaminating phospholipids as these were separated from MDHM by HPLC.

DISCUSSION

The main aspect of this work is the identification of the macrophage-activating principle MDHM from *M. fermentans*. All evidence indicates that MDHM is a lipopeptide derived from a lipoprotein (or a mixture of lipoproteins) of a type first discovered covalently bound to the murein sacculus of Gram-negative bacteria (Hantke & Braun, 1973). MDHM contained Gro-Cys, Thr, and Gly and gave an IR spectrum that strongly resembled that of a synthetic lipopeptide of a comparable structure, suggesting long chain fatty acids with ester and possibly amide linkages. Also, previously observed properties of MDHM can be explained by

this proposed structure: the amphiphilic nature of MDHM, the resistance to proteinase and phospholipases, and the alkali sensitivity due to ester-linked fatty acids. Similarly, the periodate sensitivity of MDHM is explained by our finding that MDHM contains a thioether group. Such thioethers are oxidized by periodate to sulfoxides [reviewed by Madesclaire (1986)].

It is not at all clear whether all naturally occurring lipoproteins or lipopeptides are potent macrophage activators. If we limit the discussion to mollicutes, there are 23 acylated proteins in *A. laidlawii* (Nyström et al., 1992) and about 6 in *M. fermentans* (Wise et al., 1993). However, only a few bands with macrophage stimulatory activity could be isolated from SDS−PAGE-separated proteins from *M. fermentans* (Mühlradt & Frisch, 1994; Kostyal et al., 1994). It remains to be seen which structural elements are decisive in determining macrophage-stimulating activity of such lipopeptides and to what extent various lipoproteins, which occur naturally embedded in or anchored to membranes, are broken down when processed by macrophages. According to published work with synthetic lipopeptides, the nature of the fatty acid moieties has little influence on the biological activity, whereas the presence of ester-linked acyl groups is critically important (Metzger et al., 1995). The amino acid composition of these lipopeptides determines the extent of stimulation by these compounds (Hoffmann et al., 1989; Metzger et al., 1991). There is at present little evidence that any specific sequence is required for optimal biological activity. Data from synthesized lipopeptides rather suggest that the appropriate amino acids are simply needed to provide optimal solubility in the culture medium. The importance of good water solubility for in vitro biological activity of such lipopeptides is readily apparent from the finding that solubilization with octyl glucoside increased macrophage stimulatory activity of MDHM about 10-fold (Mühlradt & Frisch, 1994). The same is true for the synthetic lipopeptide [2,3-bis(palmitoyloxy)-(2-*RS*)-propyl]-*N*-palmitoyl-(*R*)-Cys-SerLys$_4$ (P. F. Mühlradt, unpublished). However, when compared under these conditions of optimal solubility, MDHM still had a higher specific activity than the water soluble [2,3-bis(palmitoyloxy)-(2-*RS*)-propyl]-*N*-palmitoyl-(*R*)-CysSerLys$_4$ so that the possibility remains that a specific amino acid sequence next to Gro-Cys results in particularly active lipopeptides. Given the notorious variability of lipoproteins in mycoplasmas (Wise et al., 1993; Yogev et al., 1995), it will be important to isolate clones of mycoplasmas on the basis of macrophage stimulatory capacity, isolate their lipopeptides, and compare the amino acid sequences of these. This will not be an easy task, as mycoplasmas grow poorly and give low yields. The isolation and characterization of such clones from *M. fermentans* is presently under way.

A second aspect of our work is the unambiguous proof that the murein-type lipoprotein is not only encoded by mycoplasmal DNA but also fully expressed by these organisms. While this work was in progress, another group proved by paper electrophoresis the presence of the glycerylcystein thioether derived from *Mycoplasma gallisepticum* lipoproteins (Jan et al., 1995). As discussed by others, the function of these lipoproteins may be antigenic variation [e.g. Yogev et al. (1995)] or involvement in transport [reviewed by Sutcliffe et al. (1995)].

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REFERENCES

Addeo, F., Malorni, A., & Marino, G. (1975) *Anal. Biochem. 64*, 98−99.
Braun, V., & Wu, H. C. (1994) Lipoproteins, structure, function, biosynthesis and model for protein export, in Bacterial Cell Wall (Ghuysen, J.-M., & Hakenbeck, R., Eds.) Elsevier Science, Amsterdam, London, New York, Tokyo.
Chattopadhyay, P. K., & Wu, H. C. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5318−5322.
Chedid, L., Audibert, F., Lefrancier, P., Choay, J., & Lederer, E. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2472−2475.
Christiansen, G., Mathiesen, S. L., Nyvold, C., & Birkelund, S. (1994) *FEMS Microbiol. Lett. 121*, 121−128.
Cleavinger, C., Kim, M. F., & Wise, K. S. (1994) *J. Bacteriol. 176*, 2463−2467.
Dahl, C. E., & Dahl, J. S. (1984) *J. Biol. Chem. 259*, 10771−10776.
Dudler, R., Schmidhauser, C., Parish, R. W., Wettenhall, R. E. H., & Schmidt, T. (1988) *EMBO J. 7*, 3963−3970.
Ferris, S., Wilson, H. L., Neyrolles, O., Montagnier, L., & Blanchard, A. (1995) *FEMS Microbiol. Lett. 130*, 313−320.
Fidler, I. J., Nii, A., Utsugi, T., Brown, D., Bakouche, O., & Kleinerman, E. S. (1990) *Lymphokine Res. 9*, 449−463.
Frisch, M., & Mühlradt, P. F. (1996) *Eur. J. Immunol.* (in press).
Gallily, R., Salman, M., Tarshis, M., & Rottem, S. (1992) *Immunol. Lett. 34*, 27−30.
Hantke, K., & Braun, V. (1973) *Eur. J. Biochem. 34*, 284−296.
Hayashi, S., & Wu, H. C. (1990) *J. Bioenerg. Biomembr. 22*, 451−471.
Hayes, R. N., & Gross, M. L. (1990) *Methods Enzymol. 193*, 237−263.
Herbelin, A., Ruuth, E., Delorme, D., Michel-Herbelin, C., & Praz, F. (1994) *Infect. Immun. 62*, 4690−4694.
Hoffmann, P., Heinle, S., Schade, U. F., Loppnow, H., Ulmer, A. J., Flad, H.-D., Jung, G., & Bessler, W. (1988) *Immunobiology 177*, 158−170.
Hoffmann, P., Wiesmüller, K.-H., Metzger, J., Jung, G., & Bessler, W. G. (1989) *Biol. Chem. Hoppe-Seyler 370*, 575−582.
Issartel, J.-P., Koronakis, V., & Hughes, C. (1991) *Nature 351*, 759−761.
Jan, G., Fontenelle, C., Le Hénaff, M., & Wróblewski, H. (1995) *Res. Microbiol. 146*, 739−750.
Kita, M., Ohmoto, Y., Hirai, Y., Yamaguchi, N., & Imanishi, J. (1992) *Microbiol. Immunol. 36*, 507−516.
Kostyal, D. A., Butler, G. H., & Beezhold, D. H. (1994) *Infect. Immun. 62*, 3793−3800.
Loewenstein, J., Rottem, S., & Gallily, R. (1983) *Cell. Immunol. 77*, 290−297.
Lowry, O. H., Roberts, N. R., Leiner, K. Y., Wu, M. L., & Farr, A. L. (1954) *J. Biol. Chem. 207*, 1−17.
Madesclaire, M. (1986) *Tetrahedron 20*, 5459−5495.
Marshall, A. J., Miles, R. J., & Richards, L. (1995) *J. Med. Microbiol. 43*, 239−250.
McLafferty, F. W. (1983) *Tandem mass spectrometry*, John Wiley & Sons, New York.
Melchers, F., Braun, V., & Galanos, C. (1975) *J. Exp. Med. 142*, 473−482.
Metzger, J., Wiesmüller, K.-H., Schaude, R., Bessler, W. G., & Jung, G. (1991) *Int. J. Pept. Protein Res. 37*, 46−57.
Metzger, J. W., Beck-Sickinger, A. G., Loleit, M., Eckert, M., Bessler, W. G., & Jung, G. (1995) *J. Pept. Sci. 3*, 184−190.
Mizel, S. B., Oppenheim, J. J., & Rosenstreich, D. L. (1978) *J. Immunol. 120*, 1504−1508.
Mühlradt, P. F., & Schade, U. (1991) *Infect. Immun. 59*, 3969−3974.
Mühlradt, P. F., & Frisch, M. (1994) *Infect. Immun. 62*, 3801−3807.

Mühlradt, P. F., Quentmeier, H., & Schmitt, E. (1991) *Infect. Immun. 59*, 3962−3968.
Nyström, S., & Wieslander, A. (1992) *Biochim. Biophys. Acta 1107*, 39−43.
Nyström, S., Wallbrandt, P., & Wieslander, A. (1992) *Eur. J. Biochem. 204*, 231−240.
Quentmeier, H., Schmitt, E., Kirchhoff, H., Grote, W., & Mühlradt, P. F. (1990) *Infect. Immun. 58*, 1273−1280.
Ruschmeyer, D., Thude, H. J., & Mühlradt, P. F. (1993) *FEMS Immunol. Med. Microbiol. 7*, 223−230.
Ruuth, E., & Praz, F. (1989) *Immunol. Rev. 112*, 133−160.
Salman, M., Deutsch, I., Tarshish, M., Naot, Y., & Rottem, S. (1994) *FEMS Microbiol. Lett. 123*, 255−260.
Seid, R. C., Jr., Smith, P. F., Guevarra, G., Hochstein, H. D., & Barile, M. F. (1980) *Infect. Immun. 29*, 990−994.
Sher, T., Yamin, A., Rottem, S., & Gallily, R. (1990) *J. Natl. Cancer Inst. 82*, 1142−1145.
Staber, F. G., Gisler, R. H., Schumann, G., Tarcsay, L., Schläfli, E., & Dukor, P. (1978) *Cell. Immunol. 37*, 174−187.
Stuehr, D. J., & Marletta, M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 7738−7742.
Sutcliffe, I. C., & Russell, R. R. B. (1995) *J. Bacteriol. 177*, 1123−1128.
Takema, M., Oka, S., Uno, K., Nakamura, S., Arita, H., Tawara, K., Inaba, K., & Muramatsu, S. (1991) *Cancer Immunol. Immunother. 33*, 39−44.
Walther, H., Schlunegger, U. P., & Friedli, F. (1987) *Biomed. Environ. Mass Spectrom. 14*, 229−233.
Weiner, S., & Tishbee, A. (1981) *J. Chromatogr. 213*, 501−506.
Wise, K. S., & Kim, M. F. (1987) *J. Bacteriol. 169*, 5546−5555.
Wise, K. S., Kim, M. F., Theiss, P. M., & Lo, S.-C. (1993) *Infect. Immun. 61*, 3327−3333.
Yang, G., Coffman, F. D., & Wheelock, E. F. (1994) *J. Immunol. 153*, 2579−2591.
Yogev, D., Watson-McKown, R., Rosengarten, R., Im, J., & Wise, K. S. (1995) *J. Bacteriol. 177*, 5636−5643.

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