Isolation, Structure Elucidation, and Synthesis of a Macrophage Stimulatory Lipopeptide from *Mycoplasma fermentans* Acting at Picomolar Concentration

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Summary

Macrophages are typically stimulated by components of microbial cell walls. Surprisingly, cell wall–less mycoplasmas can also very efficiently stimulate macrophages. We showed recently that mycoplasma-derived lipopeptides constitute the active principle. We have now isolated a clone of *Mycoplasma fermentans* expressing mainly one macrophage-stimulating lipopeptide. This lipopeptide was detergent-extracted and isolated by reversed-phase high-performance liquid chromotography, using nitric oxide release from C3H/HeJ mouse macrophages as bioassay for detection. In contrast to "conventional" bacterial lipoproteins, this lipopeptide had a free NH_2 terminus. Amino acid composition, sequence, and the molecular weight of 2,163.3 are consistent with the following structure: S-(2,3-bisacyloxypropyl)cysteine-GNNDESNISFKEK with one mole C16:0, and a further mole of a mixture of C18:0 and C18:1 fatty acid per lipopeptide molecule. The sequence could not be found in either the protein identification resource nor the Swiss Prot data bank. We named this 2-kD lipopeptide, macrophage-activating lipopeptide-2 (MALP-2). Synthetic dipalmitoyl MALP-2 and mycoplasma-derived MALP-2 were compared with the bioassay. Both lipopeptides showed an identical dose dependency with a half-maximal response at 10^{-11} M concentration. MALP-2 may be one of the most potent natural macrophage stimulators besides endotoxin.

Apart from being a nuisance in cell culture, where mycoplasmas cause a wide spectrum of direct or indirect effects, in particular on immune cells (for review see reference 1, for more recent work see references 2–4), infection with these cell wall–less microorganisms may be associated with clinical symptoms stretching from nongonococcal urethritis (5, 6) to rheumatoid arthritis (for review see references 7 and 8, and most recent evidence in reference 9) and AIDS (10, 11). Most, if not all, events underlying these symptoms are inflammatory. It is likely that mycoplasma-mediated release of proinflammatory cytokines from macrophages is involved. The general importance in human disease of cytokine-inducing bacterial compounds, which were named bacterial modulins, and the specific involvement of cytokines in the pathogenesis of HIV-induced disease was acknowledged in two recent reviews (12, 13). Often macrophage activation was at first not recognized as such, but detected through indirect effects on B or T lymphocytes, as exemplified by the *Escherichia coli* murein lipoprotein which was first described as a B cell mitogen (14), or the mycoplasma-derived macrophage activator MDHM which was originally discovered as an inducer of CTLs (2, 15), respectively.

Macrophages are typically stimulated by components of the microbial cell wall such as peptidoglycan fragments (16, 17), LPS (18, 19), lipoteichoic acid (20), and bacterial lipoproteins (21). Surprisingly, cell wall–less mycoplasmas can also very efficiently stimulate macrophages (22). With the one notable exception of *Mycoplasma arthritidis*, a strain which produces a well-characterized protein with superantigen properties (23), the biochemical nature of putative macrophage activators from mycoplasmas is not entirely clear, and it was a matter of controversy which mycoplasmal components were responsible for this stimulation (24–28). Three independent studies recently reported that fractions containing or enriched in lipoproteins from *Mycoplasma fermentans* (27), *Mycoplasma arginini* (26), or yet other mycoplasma species (28) show macrophage stimulatory activity (MSA)[1]. However, no amino acid sequences were given, nor could it be excluded that other contaminating compo-

[1]*Abbreviations used in this paper:* Fmoc, fluorenylmethoxycarbonyl; MALDI, matrix-associated laser desorption/ionization time of flight; MALDI-MS, MALDI mass spectroscopy; MALP-2, 2-kD macrophage-activating lipopeptide; MSA, macrophage stimulatory activity; NO, nitric oxide.

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nents might be the active ones. The situation is further complicated by the fact that mycoplasmas express several lipoproteins.

We were recently able to formally prove that a lipopeptide with an acylated S-(2,3-dihydroxypropyl)cysteine NH_2 terminus is the active principle in a preparation from *M. fermentans*, formerly called MDHM (for mycoplasma-derived high molecular weight material; 29). It was derived from a proteinase K–treated detergent extract and possibly resulted from a mixture of lipoproteins. This material stimulates human monocytes as well as murine macrophages to release TNF, IL-1, IL-6, prostaglandins (2, 30) and, in the case of IFN-γ–primed murine macrophages, also nitric oxide (24, 31). Furthermore, it downregulates class II MHC expression on macrophages, resulting in impaired antigen presentation to T cells (32).

It remained unresolved why lipopeptides from mycoplasmas showed MSA at concentrations that were much lower than lipoproteins from other bacterial sources, e.g., from *E. coli* (14, 21) or borrelia and treponema (33), which are typically used at concentrations of ~1 μg/ml for half-maximal response, and 10–50 μg/ml for optimal stimulation. In contrast, macrophages can be half maximally stimulated by heat-killed mycoplasmas at a concentration of 10 ng mycoplasma protein/ml (e.g., reference 32). This indicates that even crude, unseparated mycoplasma preparations show a specific activity which is higher by several orders of magnitude than that of lipoproteins from, e.g., gram-negative bacteria. We have now isolated a clone from *M. fermentans* expressing mainly one macrophage-stimulating lipopeptide that we purified. In contrast to conventional bacterial lipoproteins (see review in reference 34), this lipopeptide has a free NH_2 terminus that enabled us to determine the complete amino acid sequence. We named this compound MALP-2 for 2-kD macrophage-activating lipopeptide. MALP-2 is half maximally active at 0.02 ng/ml corresponding to 10^{-11} M concentration. MALP-2 is the natural lipopeptide with the highest specific MSA so far described. It even surpasses the synthetic lipopeptide analogue CGP-31 362 (17) in its MSA potential by two orders of magnitude (35).

Materials and Methods

Growth of Mycoplasmas, Isolation of Clones. The *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 α medium, 10% heat-inactivated newborn calf serum (Sigma, Deisenhofen, Germany), 0.5% (wt/vol) Bacto Tryptone with 5 mM fructose, and 10 mg/L of adenosine, guanosine, cytidine, uridine, 2′-deoxyadenosine, 2′-deoxyguanosine, 2′-deoxycytidine, and 2′-deoxythymidine. For isolation of clones, mycoplasmas were filtered through a 0.45-μm filter and plated on 0.7% soft agar in this medium. Single colonies were picked with the aid of a pasteur pipette under a stereo microscope and propagated further in liquid medium. Mycoplasma cultures were split 1:10 every other day until harvest, and washed with pyrogen-free saline. Mycoplasmas were kept frozen at −20°C until use.

Nitric Oxide Release Assay. MSA was determined with the nitric oxide release assay as described (24). In brief, it is performed with peritoneal exudate cells from C3H/HeJ endotoxin low-responder mice (Bomholtgaard, Rye, Denmark) in 96-well microtiter plates. Cells are simultaneously stimulated with *r*IFN-γ and a serial dilution of macrophage-activating material. After a 48 h incubation period, nitrate is reduced with nitrate reductase, and nitric oxide (NO) determined as the sum of nitrite and nitrate using Griess reagent. One U of MSA/ml is defined as the dilution of macrophage stimulator required to obtain half-maximal NO release (24).

Detergent Extraction of Macrophage-activating Material. A frozen mycoplasma suspension containing 83-mg protein (36) in 16 ml was thawed in the presence of 1 mM PMSF and 0.6 mM *N*-(α-rhamnopyranosyloxyhydroxyphosphinyl)-l-leucyl-l-tryptophane sodium salt to deactivate and inhibit serin- and metalloproteinases, respectively. The suspension was left overnight at 4°C and then extracted with 30 ml chloroform/methanol (2:1) to remove the bulk of phospholipids. The extraction was repeated three times with 20 ml organic solvent. The water phase, including insoluble material, was freed of organic solvent by rotational evaporation, and treated for 2 h at 37°C with 5,000 U benzonase (Merck, Darmstadt, Germany) in a final volume of 20 ml 0.01 M Tris Cl, pH 7.6, 1 mM $MgSO_4$. After addition of 20 ml PBS, 40 ml 100 mM *n*-octyl β-d-glucopyranoside (octyl glucoside) were added and lipophilic proteins were extracted by heating in a boiling water bath for 7 min. Insoluble material was removed by centrifugation and the supernatant solution containing the MSA was pressure dialyzed on a YM-10 filter (Amicon, Beverly, MA) diluting the inner dialysate several times with water to remove the detergent, finally concentrating the inner dialysate to 7.5 ml. This material was kept frozen until further use.

Isolation of MALP-2 by Reversed Phase HPLC. 1 ml 100 mM octyl glucoside and 100 μl 1 M $CaCl_2$ were added to the inner dialysate which was concentrated by freeze drying. The freeze-dried material was dissolved in 1 ml 0.1 M NH_4 acetate buffer, pH 6.9, applied to an SP 250/10 Nucleosil 300-7 C8 column (Macherey & Nagel, Düren, Germany), and was eluted at 40°C with a linear water/2-propanol gradient.

Determination of Total Phosphate. Total inorganic phosphate was determined after oxidative digestion as described (37).

Amino Acid Sequence Analysis. Aliquots of 2.5–10 μl were directly taken from HPLC fractions and applied to biobrene-coated, precycled glass fiber filters of a Procise sequencer (494A; Applied Biosystems, Foster City, CA), and sequenced according to the manufacturers standard gas-phase programs (38).

Amino Acid Composition Analysis. Amino acid analysis was carried out on an amino acid analyzer (420A/H, Applied Biosystems) with automated gas-phase hydrolysis (6 N HCl, 160°C, 75 min) and on-line analysis of phenylthiocarbamoyl amino acids on an 130 HPLC with 920 data system.

Matrix-assisted Laser Desorption/Ionization Time of Flight Mass Spectroscopy. MALDI-MS was performed on a Bruker REFLEX™ (Bruker-Franzen Analytik GmbH, Bremen, Germany) instrument equipped with a nitrogen laser (337 nm, 3 ns pulse). Spectra were recorded at an acceleration voltage of 20 kV. The instrument was internally calibrated with bovine insulin. The resolution was 1:500. Octyl glucoside (8 mM final concentration) was added to aliquots from the HPLC for optimal signals (39). Such samples were mixed 1:1 (vol/vol) with 100 mM α-cyano-4-hydroxycinnamic acid in 60% aqueous acetonitrile containing 0.1% TFA and allowed to dry on the stainless steel target.

Synthesis of Lipopeptides. Resin, amino acids, and reagents were purchased from Calbiochem-Novabiochem (Bad Soden, Germany). Solvents were obtained from Fluka Chemie AG (Buchs,

Switzerland) and *N*-fluorenylmethoxycarbonyl-S-2,3-bis(palmitoyloxy)-(2-RS)-propyl-(R)-cysteine (Fmoc-Dhc[Pam_2]-OH) was synthesized as previously described (40). The lipopeptide was built up using the fluorenylmethoxycarbonyl (Fmoc) protocol for solid phase synthesis on an automated synthesizer (model 433A; Applied Biosystems). A Wang-PHB-resin loaded with tert-butoxycarbonyl–protected Fmoc-lysine residue was used as the solid support. Resin substitution was 0.60 mmol/g, and 0.1 mmol of amino acid was used for each coupling. The following side chain protecting groups were used: Asn (triphenylmethyl), Asp, Glu (tert-butoxy), Ser (tert-butyl), and Lys (tert-butoxycarbonyl). Deprotection of the Fmoc–amino acid attached to the resin was accomplished using piperidine. The amino acids were coupled using 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate and hydroxybenzotriazole (HOBt). Fmoc-Dhc(Pam_2)-OH was coupled in double excess to the resin-bound *N* 13-mer peptide with diisopropylcarbodiimide/HOBt in dimethylformamide/dichloromethane (1:2) for 12 h (41). The peptide and all protecting groups were cleaved from the resin with TFA containing phenole (5%), thioanisole (5%), ethanedithiole (5%), and water (7%). The synthesis was monitored by electrospray ionization mass spectrometry on a triple–quadrupole instrument (API III TAGA X; Perkin-Elmer Sciex, Thornhill, Ontario, Canada).

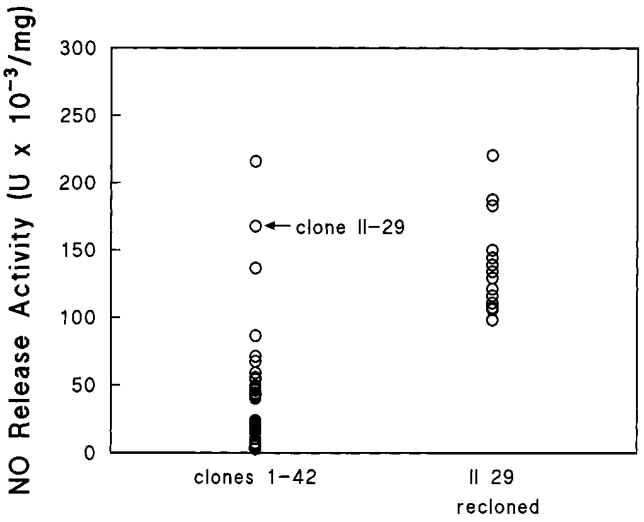

Figure 1. Macrophage-stimulatory activity of clones from *M. fermentans*. Individual colonies grown on agar were picked and then cultured in liquid medium. Mycoplasmas were harvested and MSA was extracted with hot octyl glucoside and determined in the NO release assay with IFN-γ–treated peritoneal exudate cells from LPS low-responder mice. MSA was calculated as U/mg mycoplasma protein.

Results

Different M. fermentans Clones Vary in their Macrophage-stimulatory Potential. Our earlier attempts in isolating macrophage activating material from *M. fermentans* were often frustrated by batches of mycoplasmas with low stimulatory potential. In the knowledge that (*a*) mycoplasmal macrophage stimulator is derived from lipoproteins or peptides (29), and of (*b*) the antigen variability and variation of expression of lipoproteins from mycoplasmas (42), we agar cloned mycoplasmas from their original source, a contaminated HL60 cell line, to isolate highly active clones. In total, 103 clones were picked at random, grown in liquid medium, harvested, and finally extracted with hot octyl glucoside. Macrophage stimulatory activity is extracted in this mild detergent (24). As an assay, we chose nitric oxide production by IFN-γ stimulated macrophages from LPS low responder mice. This can be used for quantitative estimation of macrophage-stimulatory activity and allows one to define a U/ml as that dilution which gives half-maximal NO production (24). A comparison of the activity of some of these clones normalized per milligram mycoplasma protein is shown in

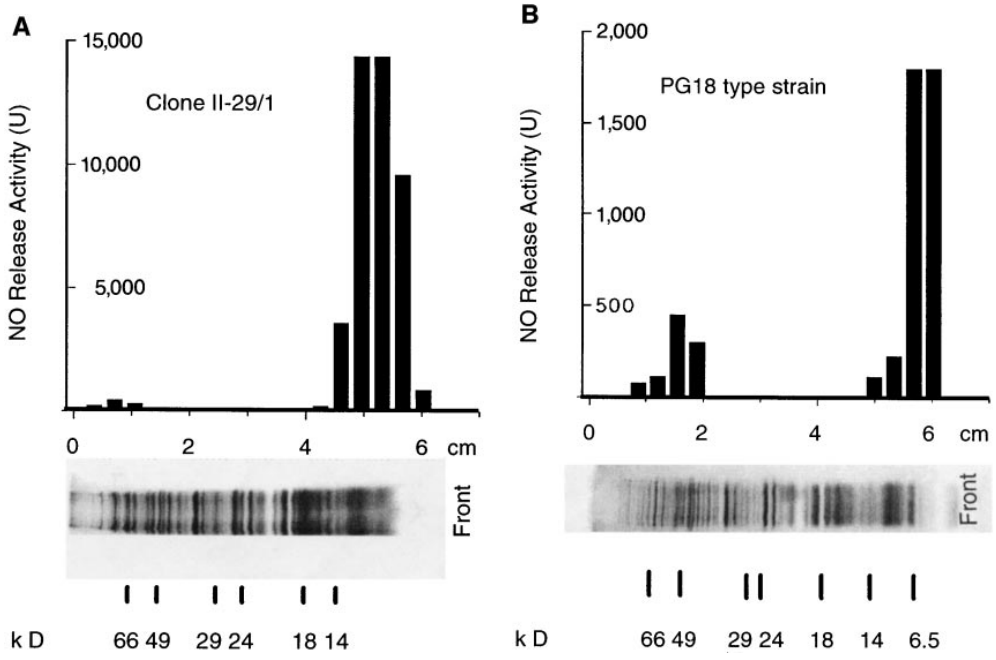

Figure 2. Comparison of silver stain and MSA in gel slices after SDS-PAGE of *M. fermentans* PG 18 type strain and clone II-29/1. Samples were run in 15% gels in the discontinuous buffer system of Lämmli under reducing conditions, applying 10 μg mycoplasma protein per lane for silver staining. 70 μg were subjected to electrophoresis on a neighboring lane. This lane was cut in 3-mm segments which were extracted in 0.3 ml hot octyl glucoside for subsequent determination of MSA.

Table 1. *Isolation of Macrophage-stimulatory Lipopeptide from M. fermentans, Clone II-29/1*

Fraction	Volume	Protein		MSA	
	ml	*mg/ml*	*mg total*	$U \times 10^{-6}$/*ml*	$U \times 10^{-6}$ *total*
Mycoplasma suspension	16	5.2	83	1.3	21
Delipidated, nuclease-treated, octyl glucoside–extracted water phase	82	0.16	13	0.32	26
Pressure dialysate/concentrate	8.2	0.41	3.4	3.8	31
HPLC peak fractions	18	ND	–	–	21

Fig. 1 and shows a variation of specific activity from 5,000 to 240,000 U/mg mycoplasma protein, i.e., by a factor of ~50. Some clones were recloned and retained their good stimulatory properties (see, e.g., clone II 29 in Fig. 1). Since the variation of MSA upon recloning was higher than can be accounted for by experimental error, we speculate that it may be due to clonal variation.

The sensitivity of the NO release assay allowed the determination of the differences in the expression of macrophage stimulatory lipoproteins and peptides from individual clones after SDS-PAGE separation by sectioning the gel and extracting the gel slices. As an example, clone II-29/1 and the type strain PG18 are compared in Fig. 2. Contrary to our expectations that a particularly active clone would exhibit a great number of stimulatory molecules with different molecular weights, clone II 29/1 (recloned from clone II 29) and similar clones in the high specific activity range showed a simple pattern with mainly one activity band at the front. Clone II 29/1 was chosen to isolate the macrophage stimulatory material.

Isolation of the Macrophage-activating Lipopeptide from M. fermentans Clone II 29/1. In our earlier studies, we primarily used proteinase K–digested macrophage stimulator, since this treatment did not destroy the activity and facilitated isolation (24). We now isolated the undegraded material by a previously established procedure with a final purification step of reversed phase HPLC (29). The extraction procedure and the yield in MSA after each step are outlined in Table 1. The elution pattern of MSA from clone II 29/1 is shown in Fig. 3. Fractions near the MSA were also tested for phospholipids by measuring inorganic phosphate. MALDI-MS was obtained from the fraction showing maximal MSA and gave a major peak at 2164.3 $[M + H]^+$ and a minor one at 2186.0 $[M + Na]^+$ (Fig. 4). After mild alkali treatment, these peaks were shifted to lower molecular weight by ~504 mass units, indicating a loss of a C16 plus C18 fatty acid (not shown). To distinguish this 2-kD lipopeptide from other lipoproteins of mycoplasma origin, we named it MALP-2.

Amino Acid Analysis and Sequence of MALP-2. The material showing maximal macrophage stimulatory activity was further analyzed for amino acid content and sequenced. Amino acid composition, sequence, and molecular weight determination are consistent with the following structure: (S-[2,3-bisacyl(C16:0/C18:0;C18:1)oxypropyl]cysteine-GNNDESNISFKEK. No NH_2-terminal amino acid was found, in

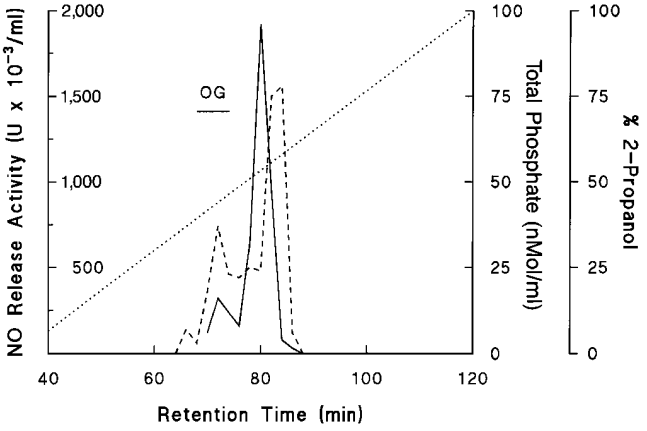

Figure 3. HPLC of octyl glucoside extracted MSA from *M. fermentans*. MSA was extracted as in Table 1. 2.6×10^7 U were applied to a 10×250–mm RP8 reversed phase column and eluted with 2-propanol. (*solid line*) MSA as determined in the NO release assay; (*dotted line*) inorganic phosphate to monitor phospholipids. The bar below *OG* shows where octyl glucoside elutes from the column.

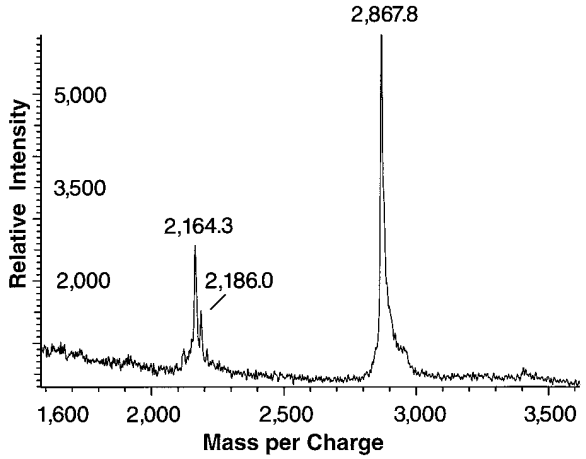

Figure 4. MALDI spectrum of HPLC-purified MALP-2. The lipopeptide gave rise to a $[M + H]^+$ ion at m/z 2164.3 and a $[M + Na]^+$ ion at m/z 2186.0. The signal at m/z 2867.8 is due to the $[M + 2H]^{2+}$ signal of the internal calibration standard bovine insulin.

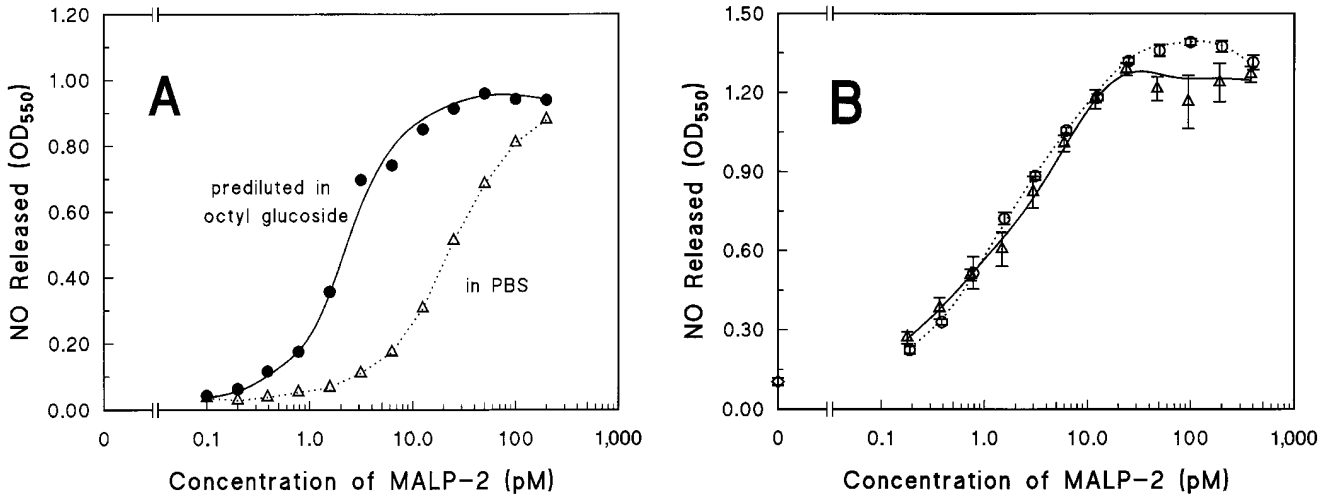

Figure 5. Concentration dependency of MALP-2 in the NO release assay. (*A*) HPLC-purified MALP-2 from *M. fermentans* clone II-29/1 prediluted in octyl glucoside (*solid line*) or PBS (*dotted line*). (*B*) HPLC-purified MALP-2 (*solid line*) and synthetic dipalmitoyl MALP-2 (*dotted line*), both prediluted in octyl glucoside. The diamond on the y-axis shows NO release of control cells with IFN-γ only. Data are the average of triplicate determinations ± standard deviation.

keeping with the previously observed instability of S-(2,3-dihydroxypropyl)cysteine upon hydrolysis. This sequence is new and could not be found in either the protein identification resource nor the Swiss Prot data bank. Noteworthy is the absence of an NH_2-terminal fatty acid substituent which is found in most bacterial lipoproteins, exemplified first by the murein lipoprotein (43). The calculated molecular weight for MALP-2 with a 1:1:1 ratio of peptide/C16:0/C18:0 would be 2164.7, as opposed to 2162.6 for a 1:1:1 ratio of peptide/C16:0/C18:1. The molecular weight of 2163.3, as determined for MALP-2 by MALDI, is compatible with a fatty acid composition of 1 mol C16:0 and a further mole of a mixture of C18:0 and C18:1 per MALP-2 molecule.

Specific Activity of MALP-2 and Comparison with Synthetic MALP-2. A dose response curve of the same HPLC-purified sample of MALP-2 that was used for amino acid analysis and whose content of MALP-2 was therefore exactly known is shown in Fig. 5. The dose response curve was determined in the presence and absence of octyl glucoside (Fig. 5 *A*). In the presence of this detergent, half-maximal response was seen at 0.002 ng/ml corresponding to 10^{-12} M, or at 10 times higher concentrations without detergent, respectively. Synthetic dipalmitoyl MALP-2 and HPLC-purified mycoplasma-derived MALP-2 was compared in Fig. 5 *B*. To ensure maximal activity, both preparations were prediluted in octyl glucoside. They showed practically identical dose response curves.

Discussion

The data presented here show that MALP-2, the macrophage-activating substance from the *M. fermentans* clone II-29/1, is a small lipopeptide with two ester-bound, long chain fatty acids and a free NH_2 terminus. We have not yet determined the exact position of the different fatty acids, as the synthetic analogue with two ester-bound palmitic acids showed the same biological activity as the natural MALP-2, suggesting that the exact nature of the fatty acid moiety may not be decisive for the MSA. In a previous paper (29) we had shown that the NH_2 terminus of a similarly active, proteinase K–treated compound is S-(2,3-dihydroxypropyl)cysteine first discovered in Braun's murein lipoprotein (43). As this earlier isolated, proteinase K–treated material was obtained from uncloned mycoplasmas and was possibly derived from more than one parent lipoprotein, it represented a mixture of lipopeptides.

It has been amply documented, in particular with synthetic analogues of the *E. coli* murein lipoprotein and peptides derived from it, that such synthetic lipopeptides constitute potent macrophage and B cell activators (21, 41, 44, 45). Moreover, synthetic conjugates consisting of lipopeptides with T helper cell and CTL epitopes from viral or bacterial proteins are efficient low molecular weight vaccines with a built-in adjuvant principle (46–48) capable of in vivo priming of virus-specific CTLs (49, 50). It is still surprising that the mycoplasma-derived MALP-2 and its synthetic analogue are half maximally active at concentrations as low as 10^{-11} M, i.e., at working concentrations in the same order of magnitude as those of lymphokines. This working concentration can still be lowered by a factor of 10 by taking care of optimal solubility through predilution in detergent (Fig. 5 *A*).

A wealth of information about which particular moieties of the lipopeptides are functionally important has been forthcoming from syntheses and assays of various analogues. Thus the presence of both ester-bound fatty acids is a prerequisite for biological activity, whereas the amide-bound fatty acid was found to be dispensable (41, 45). In fact, a free amino group at the NH_2 terminus was earlier shown to lead to very efficient stimulatory compounds (41).

Naturally occurring lipoproteins with a free amino group have previously been detected only in *Rhodopseudomonas viridis* (41). They may, however, be rather common in mycoplasmas, since, at least in *Mycoplasma pneumoniae*, whose genome has been completely sequenced, the *N*-acyltransferase gene was not detected (51). Thus, it can be expected that other lipoproteins or peptides of mycoplasmal origin will show similarly high MSA as MALP-2, as long as they contain both ester-bound fatty acids and as long as they are equally soluble. Solubility in aqueous media will, of course, depend on the amino acid composition and sequence. The importance of optimal solubility is also emphasized in Fig. 5 where MSA of MALP-2 in PBS was compared with that of detergent-solubilized MALP-2. The *M. fermentans* PG18 type strain did indeed show a second band of MSA with lower electrophoretic mobility as well as stimulatory material with the migratory properties of MALP-2 (Fig. 2). At this stage of our investigations, it is still unclear whether MALP-2 stems from such a higher molecular weight precursor or what determines its expression or appearance. An answer to these questions may arise from genetic experiments.

One important aspect of MALP-2 and other mycoplasmal lipoproteins or peptides is their probable role as pathogenicity factors in septic arthritis. Many bacteria (staphylococci, streptococci, salmonella, yersinia, mycobacteria, borrelia, etc.) can cause septic arthritis. There is no doubt that mycoplasmas are also arthritogenic in several animal species. Whether this also applies to humans is still under debate, although evidence for mycoplasmas in human arthritic joints (9, 52) strongly supports this possibility. It is almost certain that microorganisms cause macrophage activation in the affected joints which, in the case of mycoplasmal arthritis, is likely to be due to the macrophage stimulatory properties of lipoproteins that may in fact undergo antigenic variation during a prolonged infection (51). It is interesting in this context that lipoproteins from borrelia causing Lyme disease are also potent macrophage activators (33).

A second aspect could be the increasing awareness of the presence of highly active MSA in mycoplasma-contaminated cell cultures and in products from such cultures, e.g., mAbs. Although most researchers are very cautious about LPS contaminations, the fact that >30% of cells deposited in cell culture collections are mycoplasma positive indicates that the effects of contamination with mycoplasmas and their products are possibly underestimated.

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Note added in proof. While this work was submitted, we became aware of the study by R.E. Hall, S. Agarwal, D.P. Kestler, J.A. Cobb, K.M. Goldstein, and N.S. Chang, who reported the cloning of a MALP-2–related protein in the *Biochemical Journal* (Hall, R.E., et al. 1996. *Biochem. J.* 319:919–927).

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