

## Induction of Cytokines and Chemokines in Human Monocytes by *Mycoplasma fermentans*-Derived Lipoprotein MALP-2

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**Bacterial infections are characterized by strong inflammatory reactions. The responsible mediators are often bacterially derived cell wall molecules, such as lipopolysaccharide or lipoteichoic acids, which typically stimulate monocytes and macrophages to release a wide variety of inflammatory cytokines and chemokines. Mycoplasmas, which lack a cell wall, may also stimulate monocytes very efficiently. This study was performed to identify mycoplasma-induced mediators. We investigated the induction of cytokines and chemokines in human monocytes exposed to the *Mycoplasma fermentans*-derived membrane component MALP-2 (macrophage-activating lipopeptide 2) by dose response and kinetic analysis. We found a rapid and strong MALP-2-inducible chemokine and cytokine gene expression which was followed by the release of chemokines and cytokines with peak levels after 12 to 20 h. MALP-2 induced the neutrophil-attracting CXC chemokines interleukin-8 (IL-8) and GRO- $\alpha$  as well as the mononuclear leukocyte-attracting CC chemokines MCP-1, MIP-1 $\alpha$ , and MIP-1 $\beta$ . Production of the proinflammatory cytokines tumor necrosis factor alpha and IL-6 started at the same time as chemokine release but required 10- to 100-fold-higher MALP-2 doses. The data show that the mycoplasma-derived lipopeptide MALP-2 represents a potent inducer of chemokines and cytokines which may, by the attraction and activation of neutrophils and mononuclear leukocytes, significantly contribute to the inflammatory response during mycoplasma infection.**

Inflammatory reactions observed after many, if not all, bacterial infections are induced by bacterially derived molecules. An increasing number of these microbial compounds, commonly termed bacterial modulins (8), have been shown to be powerful activators of monocytes and macrophages and potent inducers of proinflammatory cytokines and chemokines. Most bacterial modulins are cell wall components. However, also preparations from mycoplasmas can powerfully stimulate macrophages/monocytes (2, 6, 15, 33, 34), although these microorganisms lack a cell wall. Recently, several reports demonstrated that crude fractions of lipoproteins derived from different mycoplasma strains showed macrophage-stimulatory activities by inducing the production of proinflammatory cytokines (9, 16, 26). In a more detailed analysis, the lipopeptide MALP-2 (macrophage-activating lipopeptide 2), recently isolated from a clone of *Mycoplasma fermentans*, has been shown to induce proinflammatory cytokines as well as nitric oxide release from mouse peritoneal macrophages (23). The structure was determined to be S-[2,3-bisacyloxypropyl]cysteine-GNNDENISFKKEK, with 1 mol of C<sub>16:0</sub> and a further mol of a mixture of C<sub>18:0</sub> and C<sub>18:1</sub> fatty acid per lipopeptide molecule (23). MALP-2 is probably derived from the larger lipoprotein MALP-404 by posttranslational cleavage (33). Such lipid modifications similar to that in Braun's prolipoprotein, which carries three fatty acids (32), are not limited to *M. fermentans* but have also been shown to occur in *M. hyorhinis* (23a).

Infections with mycoplasmas are associated with several diseases in animals and humans and are clinically relevant in cases of atypical pneumonia or chronic inflammatory syndromes such as arthritis (1, 4, 35), nongonococcal urethritis (10, 12),

and AIDS (18, 19). Moreover, mycoplasmas have been demonstrated to exert various effects on immune cells, and contamination of cell culture systems with these bacteria can often result in misinterpretation of experimental data (31).

This study was performed to examine in detail the activation of human monocytes by the *M. fermentans*-derived membrane compound MALP-2 by focusing on its capacity to cause chemokine release. Recent work of others (7, 27) has shown that synthetic MALP-2 can cause release of proinflammatory cytokines. Our results, obtained with natural MALP-2, extend these data and show that MALP-2 at low concentrations stimulated the enhanced production of several chemokines and proinflammatory cytokines. Thus, mycoplasma-derived lipopeptides seem to play a major role in attraction and immigration of immune cells into the sites of inflamed tissue during natural (30) and experimental (11, 13, 17) mycoplasma infections.

### MATERIALS AND METHODS

**Cell preparation and culture.** Human monocytes were prepared from the buffy coat of healthy blood donors provided by the Department of Transfusion Medicine, University of Marburg, Marburg, Germany, as described previously (36). Briefly, peripheral blood mononuclear cells were isolated by density gradient centrifugation over Ficoll-Hypaque. Thereafter, monocytes were further enriched by counterflow centrifugation to a purity of >95% as determined by fluorescence-activated cell sorting analysis using fluorescein isothiocyanate-labeled anti-CD14 (Immunotech, Hamburg, Germany) (41) or nonspecific esterase staining. Neutrophils used for chemotaxis experiments were separated from erythrocytes by sedimentation in 1.5% dextran and subsequent hypotonic lysis of the remaining erythrocytes as previously described (37, 38).

The monocytes were plated in 24-well tissue culture plates ( $0.75 \times 10^6$ /ml) or 60-mm-diameter tissue culture dishes ( $10^7$ /7 ml) (Falcon primary; Becton Dickinson, Paramus, N.J.) and cultured in RPMI 1640 supplemented with 2 mM glutamine, 25 mM HEPES, 1% (vol/vol) nonessential amino acids, 2 mM pyruvate, 50 U of penicillin per ml, 50  $\mu$ g of streptomycin per ml (all from Biochrom KG, Berlin, Germany), and 5% (vol/vol) heat-inactivated human AB serum (Sigma, Munich, Germany). After 1 h at 37°C and 5% CO<sub>2</sub>, nonadherent cells were removed by washing once with prewarmed phosphate-buffered saline (PBS;

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with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ; Biochrom KG). The adherent cells were cultured in medium supplemented with 2% (vol/vol) AB serum for 20 to 24 h.

**Stimulation of monocytes.** After addition of fresh medium (containing 2% AB serum), monocytes were stimulated with various amounts of MALP-2 or lipopolysaccharide (LPS; 10 ng/ml) (from *E. coli* O127:B8; Difco, Detroit, Mich.) for the indicated time periods. Thereafter, supernatants or cell lysates were harvested and stored at  $-70^{\circ}\text{C}$  until further use. MALP-2 was isolated from the *M. fermentans* clone II-29/1 by detergent extraction followed by reversed-phase high-performance liquid chromatography (23). One unit of MALP-2 is defined as the amount giving half-maximal stimulation of nitric oxide generation by mouse peritoneal exudate cells (21); 1 U of MALP-2 of this particular lot corresponds to about 2 pg/ml, corresponding to  $10^{-12}$  M. MALP-2 was kept in a stock solution of  $3.5 \times 10^6$  U/ml in 50% 2-propanol in water in the presence of 10 mM octyl glucoside (ODG). This stock solution was diluted 1:10 with 25 mM ODG and kept at  $37^{\circ}\text{C}$  for 30 min until further dilutions in RPMI<sup>SUP</sup>. The maximal final concentrations of the detergents used were 23.5  $\mu\text{M}$  for ODG and 0.005% for 2-propanol. In mock experiments, these concentrations did not affect chemokine or cytokine release.

All reagents used for cell culture were essentially free of contaminating endotoxin as determined by the *Limulus* amoebocyte lysate test (detection limit,  $<10$  pg/ml; BioWhittaker, Inc., Walkersville, Md.) and the absence of spontaneous cytokine and chemokine production.

**Determination of chemokines and cytokines.** Chemokine and cytokine release was determined by specific sandwich enzyme-linked immunosorbent assays (ELISAs) developed in our laboratory (39). Briefly, 96-well microtiter plates (Maxisorp; Nunc, Wiesbaden, Germany) were coated with a monoclonal antibody in PBS specific for interleukin-8 (IL-8; IC Chemikalien, Ismaning, Germany), GRO- $\alpha$  (Sigma), MCP-1, IL-6, tumor necrosis factor alpha (TNF- $\alpha$ ) (all from PharMingen, Hamburg, Germany), or MIP-1 $\alpha$  and MIP-1 $\beta$  (both from R&D Systems, Wiesbaden, Germany). Plates were blocked with 2% bovine serum albumin in PBS. Aliquots of culture supernatants (100  $\mu\text{l}$ /well) were incubated at room temperature for 1 h. After three washes with 0.05% Tween 20 in PBS, a specific polyclonal antibody was added in the same buffer and incubated at room temperature for another hour. The polyclonal antibodies were purchased from IC Chemikalien (IL-8), R&D Systems (GRO- $\alpha$ , MIP-1 $\alpha$ , and MIP-1 $\beta$ ), or PharMingen (MCP-1, IL-6, and TNF- $\alpha$ ). Detection was performed with a peroxidase-conjugated third antibody (donkey anti-goat or donkey anti-rabbit; both from Dianova, Hamburg, Germany) or a streptavidin-POD conjugate (Boehringer Mannheim, Mannheim, Germany) and subsequent conversion of *o*-phenylenediamine dihydrochloride substrate (Sigma). The optical density of the samples was determined photometrically at 490 nm and plotted against a standard curve performed with the respective recombinant chemokines and cytokines (purchased from IC Chemikalien [IL-8, MCP-1, and MIP-1 $\alpha$ ], R&D Systems [GRO- $\alpha$  and MIP-1 $\beta$ ], PharMingen [TNF- $\alpha$ ], or PBH, Hannover, Germany [IL-6]). The sensitivities of the established ELISAs were  $<20$  pg/ml for IL-8, MCP-1, MIP-1 $\beta$ , and IL-6,  $<50$  pg/ml for GRO- $\alpha$ , and  $<100$  pg/ml for TNF- $\alpha$  and MIP-1 $\alpha$ . Intra- and interassay variances were less than 5%.

**RNA preparation and Northern blot analysis.** Total RNA was prepared by a modified guanidine thiocyanate method as previously described in detail (40). Two micrograms of total RNA was denatured by glyoxal-dimethyl sulfoxide treatment and separated on 1% agarose gels. The RNA was capillary blotted by  $10\times$  SSC ( $1\times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate) to a positively charged nylon membrane (Boehringer Mannheim). After UV cross-linking, hybridization was performed under continuous rotation in a hybridization oven (Biotetra, Göttingen, Germany). As already reported in detail (40), the membranes were hybridized with digoxigenin (DIG)-labeled antisense riboprobes overnight under highly stringent conditions in 50% formamide at  $68^{\circ}\text{C}$ . Bound DIG-labeled riboprobes were visualized nonradioactively by using a DIG nucleic acid detection kit (Boehringer Mannheim) and CDP-Star chemiluminescence substrate (Tropix, Bedford, Mass.; distributed by Serva, Heidelberg, Germany).

**Generation and labeling of the riboprobes.** Probes (300 to 400 bp long) corresponding to human IL-8, MCP-1, MIP-1 $\alpha$ , and TNF- $\alpha$  were generated by reverse transcription-PCR and subsequent cloning of the respective PCR products. One microgram of total RNA from LPS-stimulated human monocytes was oligo(dT) primed and reverse transcribed with Superscript II reverse transcriptase (Life Technologies, Eggenstein, Germany). The cDNA was amplified by specific forward and reverse primers containing artificial restriction sites at their 5' ends by SuperTaq DNA polymerase (Stehelin, Basel, Switzerland). The amplified DNA was cloned into pCRII of a TA cloning kit as instructed by the manufacturer (Invitrogen, Leek, The Netherlands). The specificity of the inserts was confirmed by sequencing. DIG-labeled sense and antisense riboprobes were generated with SP6 or T7 RNA polymerase with a DIG-RNA labeling kit (Boehringer Mannheim), using 1  $\mu\text{g}$  of linearized vector as a template. Labeling efficiency was examined by dot blot analysis.

**Chemotaxis assay.** Cell migration was assayed in quadruplicate, using a 48-well microchemotaxis chamber technique (Neuro Probe, Bethesda, Md.) as previously described in detail (41). Culture supernatants from human monocytes exposed to MALP-2 or LPS were assayed for chemotactic activities as follows. Serial dilutions (27  $\mu\text{l}$ ) of the cell culture supernatant medium were placed into the lower chamber. After separation of the two compartments by polycarbonate filters, the upper chamber was filled with 50  $\mu\text{l}$  of freshly prepared monocytes or neutrophils ( $2 \times 10^6$  cells/ml). Monocyte migration was evaluated by using

polyvinylpyrrolidone-free filters with 5- $\mu\text{m}$ -diameter pores. The chamber was incubated at  $37^{\circ}\text{C}$  in air with 5%  $\text{CO}_2$  for 1 h. At the end of the incubation, filters were removed, fixed in methanol, and stained with hematoxylin (Sigma). The total number of migrated monocytes per well was densitometrically calculated by a computer-assisted imaging system (Vilber Lourmat; distributed by Fröbel, Wasserburg, Germany). For neutrophil chemotaxis, a polyvinylpyrrolidone-containing filter with 3- $\mu\text{m}$ -diameter pores was used to prevent adherence of the migrated cells. The number of attracted neutrophils into the lower chamber was quantitated enzymatically by determining glucuronidase activity after lysis of the cells (conversion of *p*-nitrophenyl- $\beta$ -D-glucuronide) (Sigma).

## RESULTS

**Morphology of human monocytes after exposure to MALP-2.** As analyzed by light microscopy, human monocytes cultured in the presence of MALP-2 displayed dramatic morphological changes (not shown). Control cells were adherent, round cells with short lamellipods showing the typical morphology of resting monocytes. They rarely contacted each other. After stimulation with MALP-2 for 20 h, the treated monocytes were strongly adherent, exhibited a bipolar fibroblast-like morphology, and formed a complex interconnecting network with other cells. Thus, the MALP-2-treated cells developed morphological characteristics of activated monocytes. Similar morphological changes were observed after exposure to LPS, used as a positive control stimulus.

**Dose-dependent release of cytokines and chemokines after MALP-2 stimulation.** The striking morphological changes of MALP-2-treated monocytes suggested an induction of cytokines and chemokines. When human monocytes were stimulated with increasing amounts of MALP-2 for 20 h, a strong release of the proinflammatory cytokines TNF- $\alpha$  and IL-6 occurred (Fig. 1). Significantly elevated levels were seen at 35 U of MALP-2 per ml. In contrast, weak induction of the anti-inflammatory cytokine IL-10 was observed: a 10-fold-higher dose (350 U/ml) was necessary, which then induced only low levels of IL-10. Most importantly, MALP-2 stimulated monocytes to a strong release of the CXC chemokines IL-8 and GRO- $\alpha$  and the CC chemokines MCP-1, MIP-1 $\alpha$ , and MIP-1 $\beta$ . It was of particular note that compared to the induction of proinflammatory cytokines, 10- to 100-times-lower concentrations of MALP-2 were sufficient to induce significant chemokine production.

**Kinetics of cytokine and chemokine production after MALP-2 stimulation.** To investigate the kinetics of cytokine and chemokine induction, human monocytes were exposed to MALP-2 for 2, 4, 8, 14, 22, and 48 h. Cultures stimulated with LPS (10 ng/ml) were used as positive controls. The amounts of cytokines and chemokines that were released after stimulation with 350 U of MALP-2 per ml were comparable to those obtained after LPS stimulation. Since the release of the cytokine IL-6 and the chemokines GRO- $\alpha$ , MCP-1, and MIP-1 $\beta$  showed similar kinetics as TNF- $\alpha$  and the chemokines IL-8 and MIP-1 $\alpha$ , only the latter are shown (Fig. 2). As soon as 2 to 4 h after stimulation with the lipopeptide MALP-2, significantly elevated levels of TNF- $\alpha$  (Fig. 2C) and IL-6 (not shown) were found in the supernatants. The release of TNF- $\alpha$  peaked at 8 h after stimulation and declined thereafter. Over the 48-h incubation period, only very low levels of the anti-inflammatory cytokine IL-10 were detectable after stimulation with MALP-2 (data not shown). The onset of chemokine release was similar to that of the proinflammatory cytokines. Significantly elevated levels were found as soon as 2 to 4 h after MALP-2 treatment, and peak levels were detected after 14 to 22 h (Fig. 2A and B). Thereafter, the concentrations of most of the released chemokines remained elevated as shown for IL-8 in Fig. 2A; the exception was MIP-1 $\alpha$  (Fig. 2B), which showed a slight decrease similar to that for TNF- $\alpha$ .

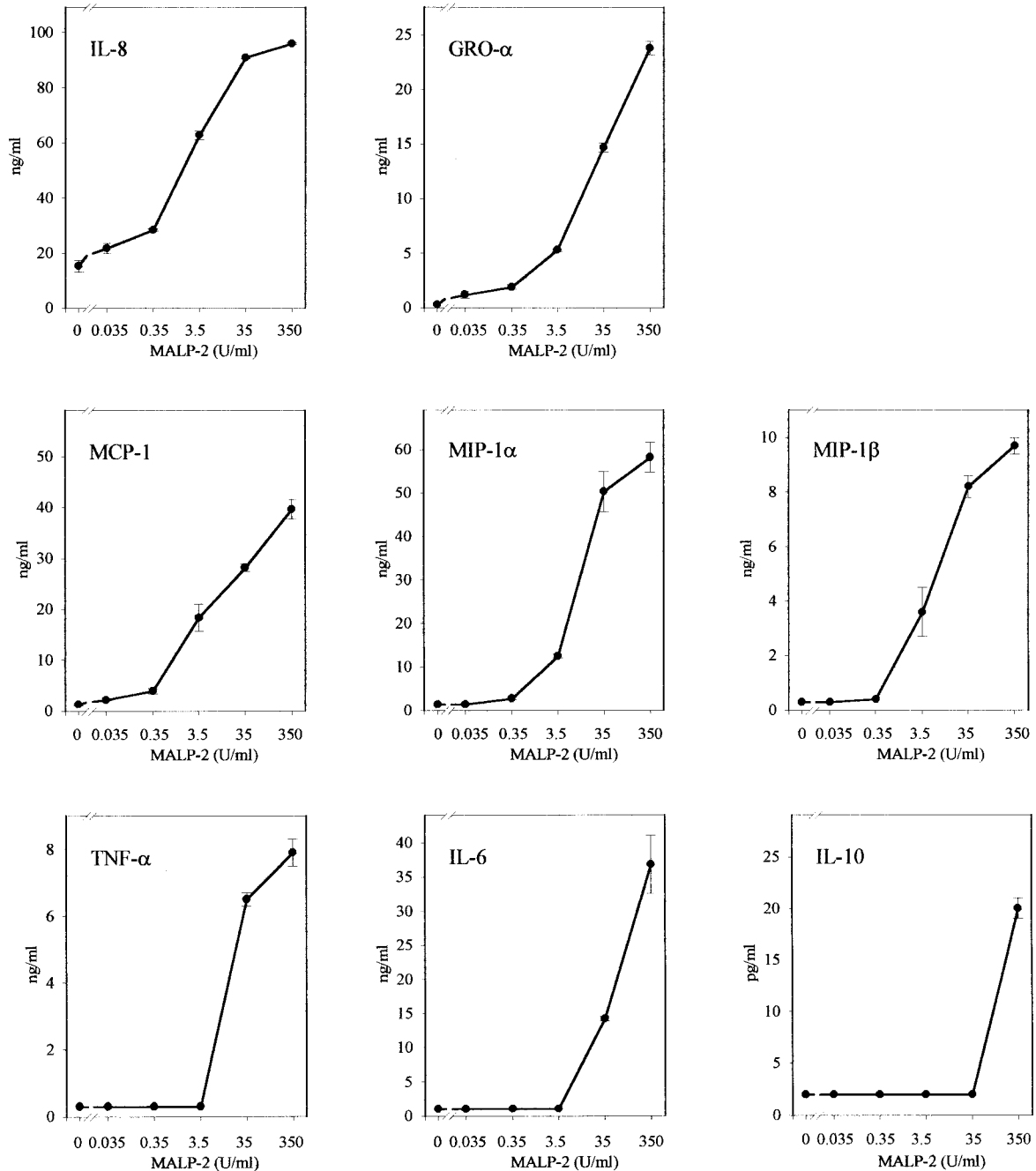


FIG. 1. Dose-dependent cytokine and chemokine release after MALP-2 stimulation. Elutriated human monocytes ( $0.75 \times 10^6/\text{ml}$ ) were either left untreated (control) or stimulated with increasing doses of MALP-2 (0.035 to 350 U/ml) for 20 h. Cytokines and chemokines were determined by specific ELISAs. Representative data from eight experiments using cells from different donors are displayed. Data are means  $\pm$  standard deviations.

**Cytokine and chemokine gene expression in MALP-2-stimulated monocytes.** To examine whether the MALP-2-stimulated secretion of cytokines and chemokines by human monocytes was due to release from stores or due to de novo synthesis, we studied gene expression by Northern blot analysis 6 h after treatment with MALP-2 and compared it to that for LPS-stimulated cell cultures. The results (Fig. 3) show clearly a strongly inducible mRNA accumulation for the cytokine TNF- $\alpha$  as well as for the chemokines IL-8, MCP-1, and MIP-1 $\alpha$ . Significantly elevated mRNA expression for TNF- $\alpha$  was

found after treatment of human monocytes with 35 U of MALP-2 per ml, which paralleled the release of TNF- $\alpha$  protein into supernatants (Fig. 1). As shown by dose-response analyses (Fig. 2B), chemokine gene expression was inducible by 10- to 100-times-lower MALP-2 concentrations compared to TNF- $\alpha$ , since 0.35 to 3.5 U of MALP-2 per ml was sufficient to induce chemokine mRNA expression. The mRNA accumulation after MALP-2 stimulation reached levels, similar to or in the case of MCP-1 even higher than levels after LPS treatment, indicating that mycoplasma-derived lipopeptide MALP-2 was generally

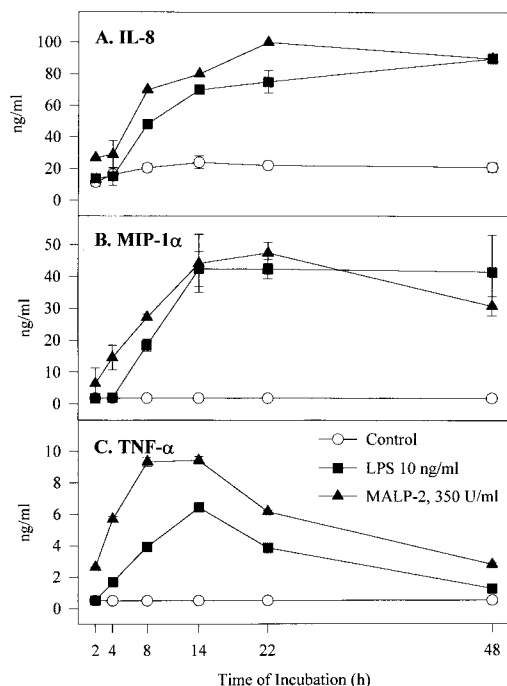


FIG. 2. Kinetics of cytokine and chemokine production after exposure of human monocytes to MALP-2. Human monocytes ( $0.75 \times 10^6/\text{ml}$ ) were either left untreated (control) or stimulated with MALP-2 (350 U/ml) or LPS (10 ng/ml) for the indicated time periods (2 to 48 h). Untreated cultures were used as a negative control. Data shown are from one of four independent experiments using cells from different blood donors.

capable of inducing a maximal expression of cytokine and chemokine genes.

**Analysis of MALP-2 inducible chemoattractant activities.** Chemotaxis assays were performed to ascertain the biological activity of MALP-2-induced chemotactic factors. Human monocytes were stimulated with MALP-2 for 20 h, and the diluted culture supernatants were analyzed in a microchemotaxis assay for monocyte- or neutrophil-specific chemotactic activities. As shown in Fig. 4, both freshly prepared monocytes and neutrophils were strongly attracted by factors released from MALP-2-stimulated monocytes. Differences from control supernatants were observed after stimulation with 35 U/ml, while 350 U of MALP-2 per ml was sufficient to elicit a full response. The numbers of attracted cells in MALP-2-conditioned culture supernatants were comparable to those responding to supernatants of LPS-stimulated monocytes. Further dilution of the culture supernatants resulted in a decreased ability to attract monocytes as well as neutrophils. When equal concentrations of MALP-2-induced supernatants were added to both sides of the polycarbonate filter, the migration of monocytes and neutrophils was arrested, indicating that migration was due to directed chemotaxis and not random chemokinesis. Neither MALP-2 nor LPS alone induced chemotaxis, and the number of migrating cells did not differ from that in experiments performed with control medium (data not shown).

## DISCUSSION

An antibacterial host defense is characterized by the recruitment of leukocytes into infected tissue and the activation of these cells for the elimination of the invaded microorganisms.

Inflammatory events are mediated by chemokines and proinflammatory cytokines which are directly induced by bacterial modulins, such as LPS, in monocytes and macrophages (14, 28, 29) or in other cells, including epithelial and endothelial cells (42) and fibroblasts (24). Also, infections with cell wall-free mycoplasmas may be associated with strong inflammatory reactions. Recently, some reports demonstrated the macrophage-stimulatory activity of mycoplasmas or mycoplasma-derived lipoprotein fractions in vitro (9, 16, 26), suggesting that mycoplasma lipoproteins may be directly responsible for the induction of cytokines and chemokines. In this study, we used MALP-2, a chemically well defined prototype of a mycoplasma-derived lipopeptide, and investigated in detail the spectrum of inflammatory mediators that were released in response to this compound.

We could clearly identify MALP-2 as a key molecule that is responsible for the known inflammatory events caused by mycoplasma infections. Monocytes exposed to this lipopeptide strongly expressed and released high levels of both cytokines and chemokines. The MALP-2-inducible activation of monocytes was accompanied by morphological changes that were very similar to those of endotoxin-stimulated monocytes. Compared to LPS stimulation, a maximal response was obtained after treatment with MALP-2 concentrations as low as 35 to 350 U/ml, which correspond to  $\sim 4 \times 10^{-10}$  M to  $\sim 4 \times 10^{-9}$  M MALP-2, respectively. Thus, the MALP-2 molecule is one of the most potent activators of human monocytes.

Our results are in line with several recent publications reporting the upregulation of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 expression after stimulation of monocytes with mycoplasma-derived membrane fractions (16, 22, 25). Gamma interferon pretreatment of murine peritoneal macrophages was reported to induce large amounts of nitric oxide when the cells were subsequently stimulated with a mycoplasma-derived high-molecular-weight material (MDHM) (22). However, the key molecule in this mixture of lipopeptides remained unknown, and the biological contribution of the various molecules could not be defined. Only recently, MALP-2 was identified as the active compound (23) and was found to be responsible for a strong expression of leukocyte attracting chemokines in vitro and the initiation of an in vivo inflammatory response in mice (5). Dose-response

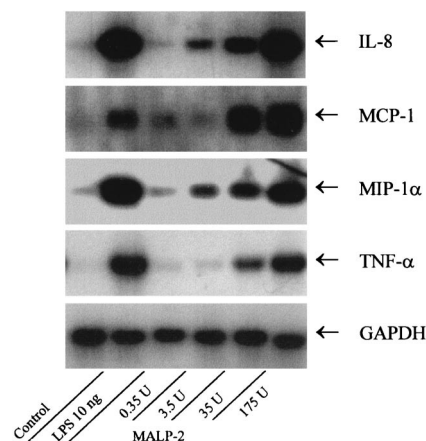


FIG. 3. Cytokine and chemokine gene expression after stimulation of human monocytes with MALP-2. Human monocytes ( $10^7/7$  ml) were either untreated (Control) or stimulated for 6 h with LPS (10 ng/ml) or increasing concentrations of MALP-2 (0.35 to 175 U/ml). Thereafter, 2  $\mu\text{g}$  of total RNA was analyzed for IL-8, MCP-1, MIP-1 $\alpha$ , TNF- $\alpha$ , and GAPDH expression by using DIG-labeled riboprobes. Results of one representative analysis of three independent experiments are shown.

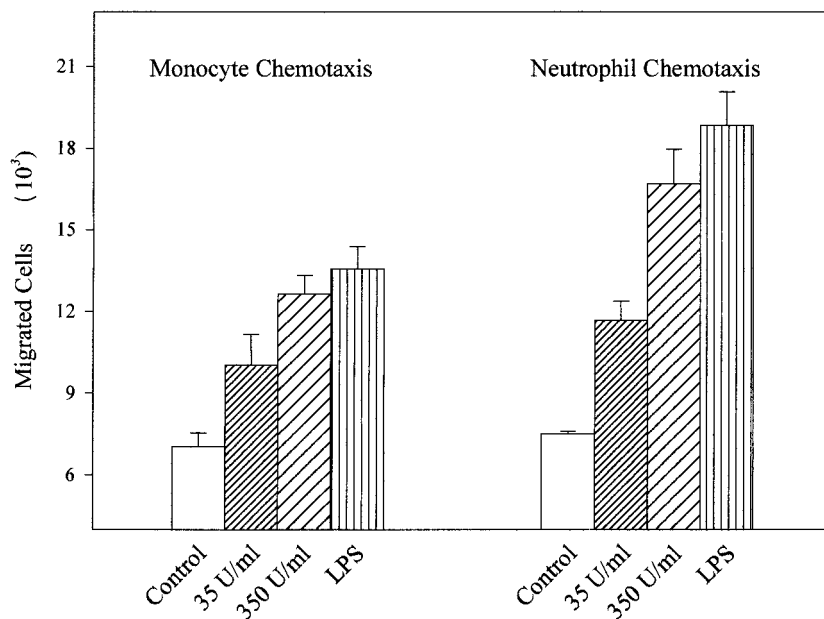


FIG. 4. Monocyte and neutrophil chemotaxis to supernatants of human monocytes stimulated with MALP-2 or LPS. Human monocytes ( $0.75 \times 10^6/\text{ml}$ ) were stimulated with MALP-2 (35 or 350 U/ml) for 20 h. The culture supernatants were diluted 1:20 or 1:50 for monocyte or neutrophil chemotaxis, respectively, and analyzed for the induced chemotactic activities in a microchemotaxis assay. Supernatants from untreated (Control) or LPS-stimulated cultures were used as negative and positive controls, respectively. The number of migrated cells was determined densitometrically (monocytes) or enzymatically (neutrophils) as described in Materials and Methods. Values represent the mean  $\pm$  standard deviations of identically prepared quadruplicate cultures.

(Fig. 1) as well as kinetic (Fig. 2) analyses clearly demonstrate that chemokine induction was independent of proinflammatory cytokine release: although the production of the cytokines TNF- $\alpha$  and IL-6 started at the same time as chemokine release, 10- to 100-fold-higher-doses of MALP-2 were required for TNF- $\alpha$  and IL-6 production. This excludes a proinflammatory cytokine-induced priming step for the initiation of chemokine release. The clearly dose dependent release of chemokines and cytokines does not seem to be a specific feature of the inducer MALP-2. Likewise, stimulation with LPS also required 10-fold-higher doses for a significant induction of the proinflammatory cytokines TNF- $\alpha$  and IL-6 compared to the induction of chemokines (data not shown). For IL-10, a delayed production may have been responsible for the low release that occurred after stimulation with high MALP-2 concentrations. However, under LPS treatment and the same incubation conditions (up to 48 h), IL-10 started to be released at around 10 h and reached maximal levels thereafter (data not shown). Thus, it appears that MALP-2 is insufficient to induce IL-10 in human monocytes. By microchemotaxis assays, we could also show that the released chemokines were biologically active and strongly attracted both freshly prepared monocytes and granulocytes (Fig. 4).

The underlying molecular mechanisms by which MALP-2 activates monocytes to proinflammatory cytokine and chemokine release are still unknown, and a receptor that mediates the MALP-2 effects has not been identified. Contamination of the MALP-2 fraction with LPS was excluded by the *Limulus* ameobocyte lysate test and by the procedure for purification of MALP-2 using reversed-phase high-performance liquid chromatography. Furthermore, a fully synthetic MALP-2 analogue with two ester-bound palmitic acids showed cytokine and chemokine-inducing capacities similar to those of the purified, naturally occurring material (data not shown). Tyrosine kinases seems to be involved in the intracellular signal transduc-

tion cascade, in that tyrosine phosphorylation is a crucial event in the mycoplasma-mediated induction of proinflammatory cytokines in THP-1 cells and human monocytes (26). Studies concerning the signal pathways utilized by MALP-2 are in progress. The observation that ester hydrolysis totally abolishes the macrophage-stimulatory activity of MALP-2 (data not shown) suggests that a direct interaction may take place between the amphipathic MALP-2 molecule and the cellular membrane (20).

In conclusion, our data offer an explanation for the leukocyte infiltration and inflammatory response after mycoplasma infection. The induction of chemokines and proinflammatory cytokines by the mycoplasma-derived lipopeptide MALP-2 appears to be the key factor for the attraction and activation of neutrophils and mononuclear cells within the infected tissue. In particular, the early induction of chemokines by traces of the powerful mycoplasma compound MALP-2 may account for the rapid influx of phagocytes and successful eradication of mycoplasmas without causing an overt, proinflammatory cytokine-based anti-infectious response.

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