

Background: Cell-wall components of Gram-positive and Gram-negative bacteria induce the production of cytokines in human peripheral blood mononuclear cells. These cytokines are the main mediators of local or systemic inflammatory reaction that can contribute to the development of innate immunity.

Aims: This study was performed to analyze the involvement of CD14 molecule in the activation of human monocytes by peptidoglycan monomer (PGM) obtained by biosynthesis from culture fluid of penicillin-treated *Brevibacterium divaricatum* NRLL-2311.

Methods: Cytokine release of interleukin (IL)-1, IL-6 and tumor necrosis factor- α from human monocytes via soluble CD14 (sCD14) or membrane-associated (mCD14) receptor using anti-CD14 monoclonal antibody (MEM-18) or lipid A structure (compound 406) was measured in bioassays.

Results: The results demonstrated that PGM in the presence of human serum might induce the monokine release in a dose-dependent manner. The addition of sCD14 at physiologic concentrations enhanced the PGM-induced monokine release, while the monokine inducing capacity of PGM in the presence of sCD14 was inhibited by MEM-18. Effects of PGM were also blocked by glycolipid, compound 406, suggesting the involvement of binding structures similar to those for lipopolysaccharide.

Conclusion: Activation of human monocytes by PGM involves both forms of CD14 molecule, sCD14 and mCD14.

Keywords: Peptidoglycan monomer, Peptidoglycan, Lipopolysaccharide, CD14, Membrane CD14, Soluble CD14, Anti-CD14 monoclonal antibody, Compound 406, Monocytes, Cytokines

The involvement of CD14 in the activation of human monocytes by peptidoglycan monomers

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Introduction

Cell wall components of Gram-negative and Gram-positive bacteria have been reported to induce inflammatory responses *in vivo*, as well as *in vitro*.^{1–5} When localized, this inflammation helps to eradicate the invading organisms at the infected site, but in severe situations the release of soluble cellular mediators and dysfunction of numerous host defense factors may result in systemic inflammation that causes organ damage, sepsis, and death. Septic shock is a classical response to severe infections due to Gram-negative bacteria and its principal component lipopolysaccharide (LPS).^{6–8} Similar events, however, may also be provoked by Gram-positive bacteria⁹ containing, in cell walls, predominantly thick peptidoglycans (PG), which may also be obtained in the soluble form (sPG), after the treatment of growing bacteria by β -lactam antibiotics.^{10,11} Chemically, PG is an alternating linked

N-acetylmuramyl and *N*-acetylglucosaminyl glycan whose residues are cross-linked by short peptides.^{10–13} Soluble PG, obtained from staphylococci, consists of disaccharide-peptide polymers linked together via 1–4 β -glycosidic bonds, without the interpeptide bridges, due to the action of penicillin.^{10–13} All these components, similar to lipoteichoic acid (LTA), lipoarabinomannan, and other glycosyl residue-containing compounds, stimulate the release of proinflammatory mediators in the host from various cell types.^{1–9} However, although it is evident that PG has, in principle, the same inflammatory activity as LPS, the knowledge on the contribution of PG in distinct inflammatory reaction, as well as the mechanisms of its signal transduction in lymphoid cells, is limited. Evidence shows that activation of cells by PG is a receptor-mediated process involving the binding of PG to membranous CD14 (mCD14),^{1–14} and to the additional signal transducing receptors, belonging to the Toll-like

receptor family.¹⁵⁻¹⁹ However, although both LPS- and PG-mediated signals seem to activate similar types of receptors, the type of signal and co-receptors may differ, resulting in different requirement of serum factors and activation of different sequences in the region of CD14.^{20,21}

Since there is still a paucity of data regarding the mechanisms leading to inflammatory response induced by Gram-positive bacteria, in spite of the fact that in recent years they have caused nosocomial infections and shock more frequently than Gram-negative bacteria,²² in the present study we tested the effects of peptidoglycan monomer (PGM), obtained by biosynthesis from culture fluids of penicillin-treated *Brevibacterium divaricatum* NRRL-2311^{23,24} on cytokine production of human peripheral blood mononuclear cells and on the involvement of soluble (sCD14) and mCD14 in these events. PGM represents a water-soluble monomeric structural subunit of the polymeric PG, consisting of β 1,4-linked *N*-acetylmuramic acid-*N*-acetyl-D-glucosamine disaccharide carrying the pentapeptid. Its immunostimulating and anti-metastatic activities were emphasized in many experimental protocols,²⁵⁻²⁸ but its action is still unclear. The data showed that PGM has a cytokine-stimulating capacity *in vitro*, leading to release of interleukin (IL)-1, IL-6, and tumor necrosis factor- α (TNF- α) in the presence of human serum or sCD14. Since the effects were blocked by anti-CD14 monoclonal antibodies (MEM-18) and by lipid A partial structure (compound 406), the data also suggest the involvement of both forms of CD14 molecules in these events.

Materials and methods

Cell wall components

PGM (*N*-acetyl-glucosaminyl- β -(1 \rightarrow 4)-*N*-acetyl-muramoyl-L-alanyl-D-isoglutaminyl-L-meso-diaminopimelyl-(D-amide)-(L)-D-alanyl-D-alanine), a disaccharide pentapeptide from Gram-positive bacteria *B. divaricatum*, was isolated and purified as described elsewhere.^{23,24} It contained less than 2.25 pg of endotoxin per mg of PGM, which was determined by the Limulus Amebocyte Lysate (LAL) assay (Haemochrom Diagnostica GmbH, Essen, Germany). LPS was isolated from *Salmonella friedenaui* and purified by the phenol-chloroform-petroleum ether method.²⁹ This preparation contained less than 0.2% of protein and nucleic acids as determined by chemical analysis.

Synthetic compound 406 (precursor Ia), a tetra-acyl partial structure of *Escherichia coli* hexa-acyl lipid A lacking dodecanoic and tetradecanoic acids, was synthesized as described previously.³⁰ Compound 406 was kindly provided by Dr S. Kusumoto, Department of Chemistry, Faculty of Science, Osaka University, Osaka, Japan.

Antibodies

The CD14-specific monoclonal antibody (murine hybridoma MEM-18 producing immunoglobulin (Ig)G1 monoclonal antibody) was kindly provided by Dr Václov Horejší, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic.³¹ Purified mouse IgG1 (MOPC21; Sigma GmbH, Deisenhofen, Germany) was used as an antibody isotype control.

Recombinant human soluble CD14

Recombinant human soluble CD14 (sCD14)³² was purified from serum-free culture supernatant of sCD14-transfected CHO cells by affinity chromatography (kindly provided by Dr F. Stelter, Institut of Immunology and Transfusion Medicine, University of Greifswald, Greifswald, Germany).

Cell isolation and induction of TNF, IL-1 and IL-6 release

Human blood from different healthy donors (permission was obtained from the Local Ethical Committee) was collected in heparin (10 U/ml). The mononuclear cells (MNC) were isolated by density-gradient centrifugation on Ficoll-Paque (Pharmacia, Uppsala, Sweden) as described elsewhere.³³ After being washed three times, MNC (4×10^6 /ml) were suspended in RPMI 1640 supplemented with 100 U/ml of penicillin and 100 μ g/ml of streptomycin (RPMI-1640-M). The cells were stimulated for 8 h at 37°C (5% CO₂) in 200 μ l cultures with an appropriate cytokine inducer in the presence or absence of 10% heat-inactivated human serum (HS) in U-bottomed microtiter plates (Greiner, Nürtingen, Germany). All cultures were carried out in triplicate. After incubation, supernatants were harvested and stored at -20°C until analyzed for IL-1, IL-6 or TNF activity. In the inhibition experiments with anti-CD14 monoclonal antibody (mAb) or compound 406, MNC were first pretreated for 1 h with these compounds on ice, after which the stimulus (PGM or LPS) was added. To investigate the effect of sCD14, PGM or LPS were first preincubated for 3 h with sCD14 and then added to MNC (4×10^6 /ml) in RPMI-1640-M without HS.

Determination of TNF, IL-1 and IL-6 bioactivity

TNF and IL-1 were determined in bioassays as described elsewhere.^{33,34} In brief, TNF cytotoxic activity in culture supernatants was measured using the TNF-sensitive L929 fibrosarcoma cell line. In the IL-1 bioassay, the proliferation-inducing activity of the culture supernatant from human foreskin dermal fibroblasts was determined.³⁴ IL-6 activity was

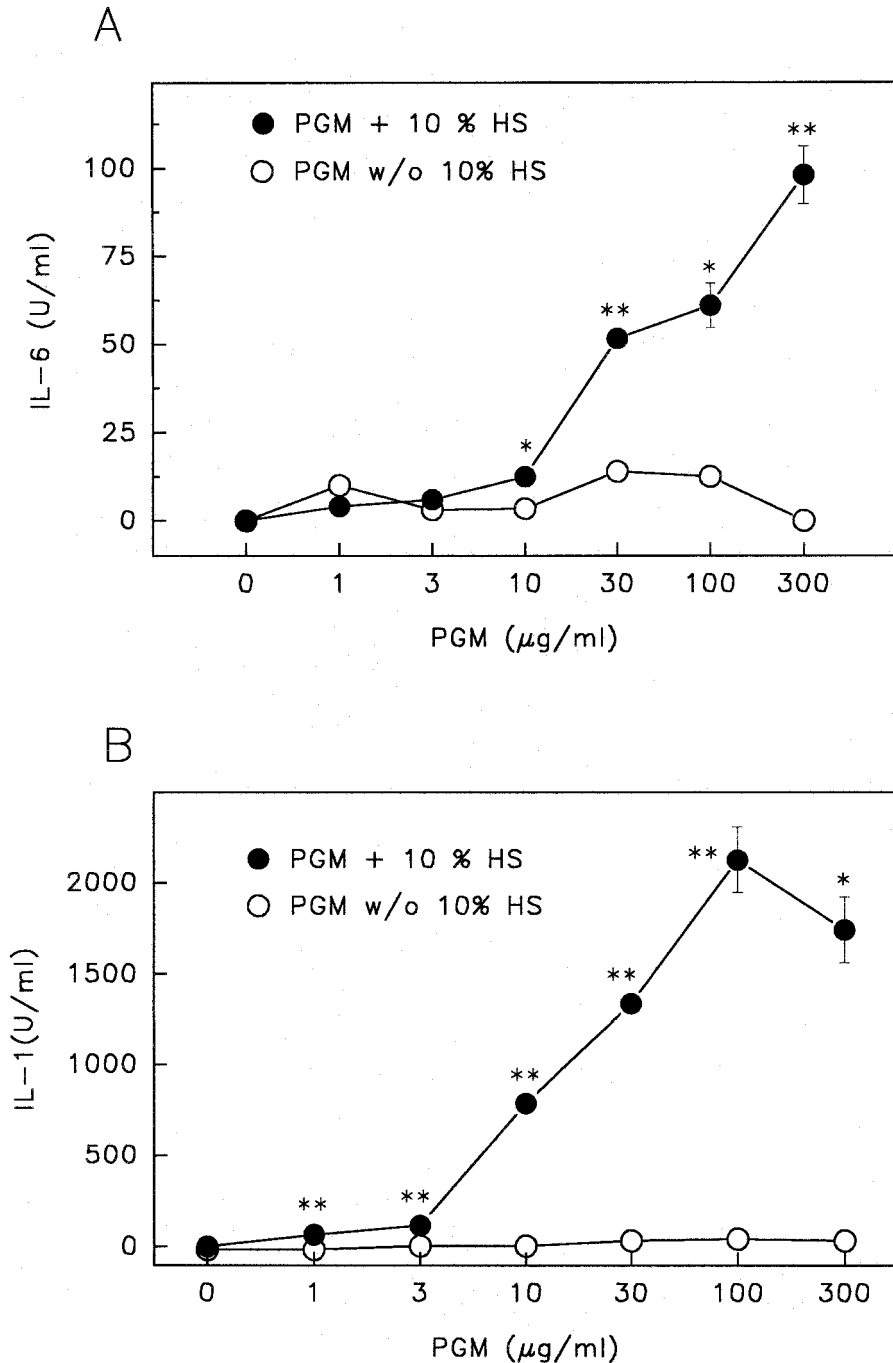


FIG. 1. Induction of IL-6 and IL-1 release from mononuclear cells (MNC) by peptidoglycan monomer (PGM) in the presence or absence of human serum (HS). MNC (4×10^6 /ml) were stimulated with increasing concentrations of PGM in the presence or absence of 10% HS for 8 h. The supernatants were harvested, and (A) IL-6 or (B) IL-1 were measured in bioassays. The data represent the mean of triplicates \pm SEM from one of three identical experiments. * $p < 0.05$, ** $p < 0.01$, PGM + 10% HS versus PGM without (w/o) 10% HS.

determined by the IL-6-dependent B9.9-3A4 cell proliferation assay.³⁵ The TNF, IL-1, and IL-6 activities in the samples were determined by probit analysis using a standard preparation of recombinant human TNF- α , IL-1 β or IL-6, respectively.³⁶ The results were calculated to units per milliliter of TNF- α , IL-1, and IL-6, respectively. The following agents did not induce release of these cytokines after

addition to monocytes: human serum, compound 406, anti-CD14 mAb (MEM-18), and human recombinant soluble sCD14.

Statistical analysis

Results were analyzed using the SigmaPlot Scientific Graphing System Version 5.0 (Jandel Corporation,

Table 1. The effects of soluble CD14 (sCD14) and anti-CD14 monoclonal antibody (MEM-18) on IL-1 release from human mononuclear cells (MNC) stimulated with peptidoglycan monomer (PGM) or lipopolysaccharide (LPS)

Stimulation of MNC in the presence of:	IL-1 release (U/ml) after stimulation with:	
	PGM	LPS
Without sCD14	166 ± 7	188 ± 8
sCD14	456 ± 5†	447 ± 9†
Anti-CD14 + sCD14	24 ± 2*	12 ± 2*
IgG1 + sCD14	445 ± 6	453 ± 7

The results are means SEM of triplicates from one out of three separate experiments.

* $p < 0.001$ versus the same substance with IgG1 + sCD14; † $p < 0.001$ versus the same substance without sCD14.

USA). The test for significance used in all cases was Student's *t*-test.

Results

PGM induces IL-6 and IL-1 release from cultured MNC

Human MNC were treated for 8 h with various concentrations of PGM in the presence or absence of 10% HS. As shown in Figure 1, PGM stimulated the release of only small amounts of IL-6 (Fig. 1A) or IL-1 (Fig. 1B) in the absence of HS. However, after the addition of 10% HS, the statistically significant ($p < 0.05$ and $p < 0.01$) dose-dependent increase in the cytokine concentrations was noticed in supernatants of PGM-stimulated MNC (Fig. 1). A similar dose-dependent effect of PGM was obtained on the secretion of TNF (data not shown), pointing to the role of sCD14 in PGM-induced release of inflammatory cytokines from human monocytes.

sCD14 increases both the PGM- and LPS-induced IL-1 release from human monocytes, and anti-CD14 mAb inhibits this process

To prove the role of sCD14 in PGM-induced release of monokines and to compare this process with that induced by LPS, PGM or LPS were pre-incubated with sCD14 for 3 h and then added to cultures of human monocytes in additional experiments. The concentration of IL-1 found in these cultures was compared with that found in cultures without added sCD14, with those treated with anti-CD14 mAb MEM-18, and with those treated with anti-IgG1 mAb. As presented in Table 1, the addition of sCD14 significantly enhanced the potential of both PGM ($p < 0.001$) and LPS ($p < 0.001$) to release the IL-1 from monocytes, and anti-CD14 mAb (significantly, $p < 0.001$) almost completely blocked this process, while an isotype control (anti-IgG1 mAb) did not block PGM- or LPS-induced cytokine production.

Anti-CD14 mAb inhibits PGM- and LPS-induced TNF release from MNC

To elucidate the potential role of mCD14, in the further experiment, we preincubated human MNC with increasing concentrations of anti-CD14 mAb (MEM-18) or anti-IgG1 mAb before the addition of PGM (Fig. 2A) or LPS (Fig. 2B). As shown in Fig. 2A,B, anti-CD14 mAb significantly blocked the TNF production in supernatants of PGM ($p < 0.01$) or LPS ($p < 0.01$) stimulated cultures, while an isotype control did not have an inhibitory effect on PGM- or LPS-induced cytokine production. This indicates that anti-CD14 mAb may abolish PGM-induced and LPS-induced TNF production.

The release of TNF from MNC induced by PGM or LPS was completely inhibited by lipid A partial structure (compound 406)

To partially define the PGM binding structures on the membrane, human MNC were pretreated with increasing concentrations of compound 406, which is a partial lipid A structure. The data showed that compound 406 was able to significantly decrease or abolish ($p < 0.001$) both PGM- and LPS-induced TNF production (Fig. 3), indicating that PGM uses similar receptor structures as LPS during the activation of human monocytes.

Discussion

In contrast to Gram-negative bacteria, the knowledge about the cytokine induction due to the adhesion of Gram-positive bacteria to human MNC is limited and concentrated mostly on the mechanisms of action of sPG, muramyl dipeptide (MDP) or LTA.^{2,5,9-13,16,19} The signaling mechanisms induced by water-soluble, monomeric glycopeptides, such as PGM, are even less known, although it has been repeatedly shown that this product might have very useful immunostimulating abilities.²⁴⁻²⁸ The data presented herein therefore represent, to our knowl-

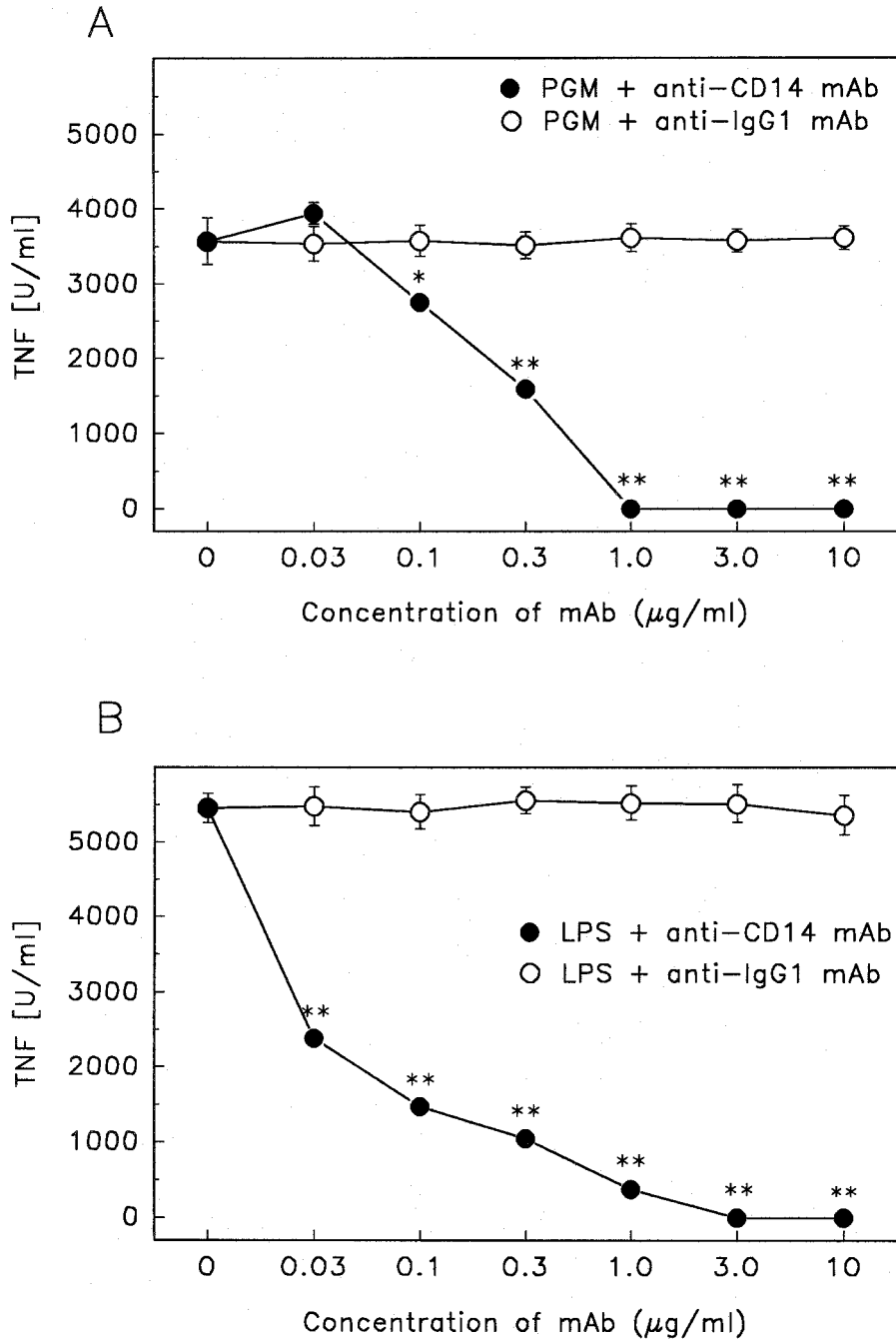


FIG. 2. Inhibition of peptidoglycan monomer (PGM)- and lipopolysaccharide (LPS)-induced tumor necrosis factor (TNF) release in mononuclear cells (MNC) by anti-CD14 monoclonal antibody (mAb) (MEM-18). MNC (4×10^6 /ml) were preincubated with increasing concentrations of anti-CD14 mAb (MEM-18) or purified mouse IgG1 (MOPC21), which was used as an antibody isotype control for 1 h at 4°C before stimulation with (A) PGM (30 µg/ml) or (B) LPS (10 ng/ml). After 8 h of incubation (in the presence of 10% human serum), supernatants were harvested and TNF activity was determined in the bioassay. Results represent the mean of triplicates \pm SEM from one of three identical experiments. * $p < 0.05$, ** $p < 0.01$, PGM + anti-CD14 mAb versus PGM + IgG1, LPS + anti-CD14 versus LPS + IgG1.

edge, the first evidence of the CD14-dependent cytokine production induced by PGM (disaccharide-pentapeptide) on human MNC. The results emphasize that HS increases PGM-induced monokine production by human monocytes (Fig. 1), and clearly show that preincubation with sCD14 enhances, while anti-CD14 mAb (MEM-18) blocks, the PGM-

induced release of IL-1 (Table 1). Furthermore, since it was found that MEM-18, similar to a lipid A partial structure (compound 406) (Fig. 3), may block the biological activity of PGM (Fig. 2), our data suggest that mCD14 is also involved in the PGM-induced cytokine release from MNC. Similar to this, unpublished data show that cytokine production in human

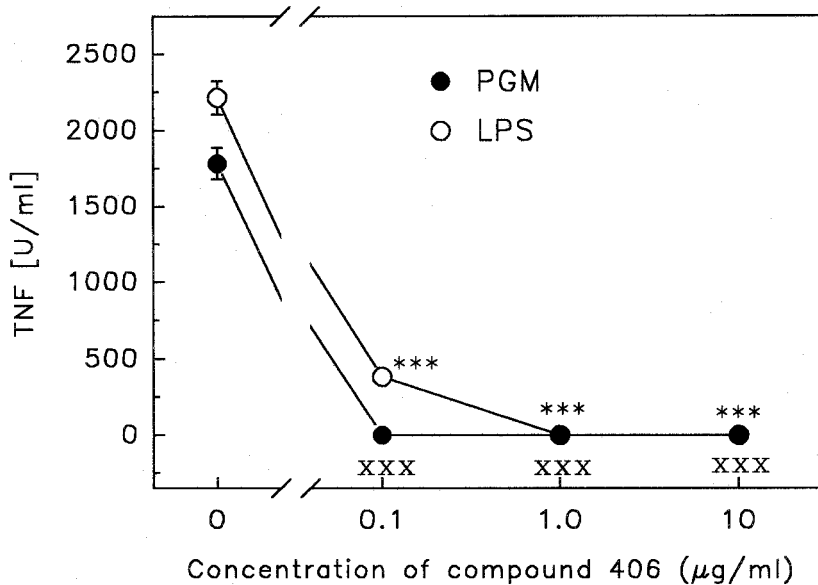


FIG. 3. Inhibition of tumor necrosis factor (TNF) release induced by peptidoglycan monomer (PGM) and lipopolysaccharide (LPS) from mononuclear cells (MNC) by the compound 406. MNC (4×10^6 /ml) were preincubated with increasing concentrations of compound 406 for 1 h at 4°C before stimulation with PGM (30 µg/ml) or LPS (10 ng/ml). After 8 h of incubation (in the presence of human serum), supernatants were harvested and TNF activity was detected in the bioassay. Results represent the mean of triplicates \pm SEM from one of three identical experiments. *** $p < 0.001$, PGM versus PGM with compound 406; xxx $p < 0.001$, LPS versus LPS with compound 406.

monocytes induced by MDP can be inhibited by anti-CD14 mAb (A.J. Ulmer, personal communication). Taken together, the data point to the similarity of action between PGM, MDP, sPG and LPS, and other bacterial products supporting the hypothesis that CD14 functions as a 'pattern recognition receptor',¹⁴ which probably recognizes shared features of all these agents. Moreover, similar to as it was shown for other Gram-positive peptidoglycans,¹⁰ the proinflammatory effects of PGM were less emphasized than that induced by LPS, since it was noticed in the presence of human serum that, for optimal cytokine production from the peripheral blood MNC, an approximately 10^5 times higher concentration of PGM than of LPS (4 log difference (µg/ml)) is required (unpublished data). The observed difference has been attempted to be explained by the existence of low affinity binding interaction between PGM and CD14 molecule,¹⁰⁻¹² as well as by the hypothesis that PG is either less active than LPS or only a part of PG is required for PBMC activation.³⁷

Our data obtained with PGM are in close agreement with results published by Weideman *et al.*,¹⁰ which show similar binding characteristics of sPG. However, in the literature there is some disagreement about the determination of structures responsible for the activation of human monocytes by products of Gram-positive bacteria. Thus, although our data show that PGM, as the hydrophilic disaccharide-pentapeptide, has similar effects as hydrophilic *N*-acetylmuramyl-L-alanyl-D-isoglutamine or

MDP, which in binding inhibition studies was also able to block the binding of fluorescein isothiocyanate-labeled sPG to human monocytes,¹⁰ in other experiments using the gel shift assay it was shown that soluble MDP did not bind to sCD14 and did not inhibit binding of sPG or LPS to sCD14.¹¹ The reason for this difference is unclear, but it was hypothesized that polymeric, aggregated or solid-phase bound PG or MDP should be formed for high affinity binding to CD14 in a gel shift assay, which might not be created after the use of very hydrophilic synthetic PG fragments.¹¹ This is also supported by our data showing that PGM also does not inhibit binding of sPG or LPS to sCD14 in the gel shift assay (unpublished data). As further support of our data, pointing to activities of soluble PGM, it was recently emphasized that digestion products of *Streptococcus pneumoniae* cell walls of a molecular weight between 600 and 15,000 might have different proinflammatory activities.³⁷ Thus, a simple stem peptide ($M_r < 750$) was 10-fold less active than undigested peptidoglycans, in contrast to tripeptides ($M_r > 1000$), which were 100-fold more potent than the native material. Moreover, while one dipeptide was inactive, two tripeptides were active, pointing to the possibility that complex branched peptides, which represent only 2% of the total material, were responsible for peptidoglycan-induced inflammation. Owing to these facts, it was proposed that, to stimulate target cells, both soluble and cross-linked peptide must be present at least three stem peptides in close proximity.³⁷

Recent studies also provided unequivocal evidence that, for cellular responsiveness to sPG, the N-terminal 151 amino acids of mCD14^{5,11} were sufficient, but the binding characteristics of hydrosoluble PGM were not fully elucidated. Our finding of dose-dependent, saturable, and anti-CD14 sensitive effects of PGM, however, directly points to involvement of CD14 (Figs. 1 and 2, and Table 1). Furthermore, since cytokine production induced by PGM was blocked by MEM-18 (Fig. 2), whose epitope is located between amino acids 51 and 64, there is also some possibility that these fragments were particularly responsible for the effects of our immunostimulating agent, similarly as it was found for sPG and LPS.¹¹ Additionally, owing to the fact that there is structural similarity between PGM and lipid A with regard to GlcNAc-MurNAc and (GlcNAc-MurNAc)₂, respectively, it could be hypothesized that active parts of PGM were hydrophilic domains, exposed on the surface and available for binding to CD14.

Taken together, although further characteristics of the binding of PGM remain to be elucidated, our data provide the first evidence that, in the induction of cytokine production in human monocytes, not only the polymeric sPG, but also the monomeric hydrosoluble PGM are involved. For optimal activation, PGM required the presence of sCD14 or other human serum components and mCD14. In the further experiments, it must be clarified whether CD14 in these events acts as a 'pattern recognition' receptor,¹⁴ an unspecific carrier molecule, or as a contributor that, together with LPS-binding protein (LBP) in a 'combinatorial' scheme, participates in recognition of these and other bacterial products.^{3,11,14}

ACKNOWLEDGEMENTS. The authors wish to thank Prof. Dr Hans-Dieter Flad for acceptance and financial support of D.M., who was a visiting scientist at the Department of Immunology and Cell Biology, Research Center Borstel, Borstel, Germany. The authors are also grateful to Prof. Dr E.Th. Rietschel and Prof. Dr A.J. Ulmer for stimulating discussion during preparation of this manuscript, as well as for permission to cite some unpublished data from their laboratory. D.M. also wishes to thank Dr Parvis Shayan for the performance of LAL tests, and Ina Goroncy, Katrin Klopfenstein, Carola Schneider and Tanja Hempel for their expert technical assistance. This work was partly supported by the grants from the Croatian Ministry of Science (numbers 006231 and 006202). For donation of PGM and its derivatives, we would like to thank the research staff of Pliva Pharmaceutical, Zagreb, Croatia.

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**Received 19 April 2001;
accepted 3 May 2001**