Structural Requirements of Synthetic Muropeptides to Synergize with Lipopolysaccharide in Cytokine Induction*

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Muropeptides contribute to the recognition of bacteria by modulating immune responses: the structural requirements for adjuvant activity were described in the seventies. During the last years, our knowledge of bacterial pattern recognition has increased dramatically and the importance of the absence of contaminations in both muropeptide preparations and other bacterial stimuli has become clear. We investigated a panel of 15 synthetic Limulus-negative muropeptides, four of them synthesized for the first time, as to their potency to synergize with lipopolysaccharide (LPS) in cytokine induction in human whole blood. No muropeptide was capable of stimulating cytokine release from human blood. However, as little as 20 nm of the muropeptides N-acetyl-muramyl-L-alanyl-D-isoglutamine (muramyl dipeptide, M(AlQ)), N-acetylmuramyl-L-alanyl-D-isoglutamine-muramyl dipeptide GM(AoQ), or C16M(AoQ), which carries a non-natural additional fatty acid, sufficed to induce an up to 3 log-order shift in tumor necrosis factor α-release in response to 100 pg/ml LPS. The release of interleukin-1β, interleukin-6, and interleukin-10 was also significantly enhanced although to a lesser extent. The synergistic effect was stereoselective with M(AoQ) being the minimal active principle. Synergy was also observed on the transcriptional level by means of real-time PCR. Smaller molecules like N-acetylmuramic acid (M), A,M, carrying a naturally occurring 1,6-anhydro-bound in M or M(A), containing only the amino acid D-alanine neither synergized with LPS nor influenced the synergy of other muropeptides with LPS. In conclusion, these data show that nanomolar quantities of muropeptides dramatically potentiate LPS-induced monocyte activation. This has implications for pyrogenicity testing and endotoxemia in patients.

The adjuvant activities of degradation products of peptidoglycan, a major constituent of the Gram-negative and Gram-positive cell wall, are well known and have been intensively characterized for decades (1). In 1974, muramyl dipeptide (MDP) was found to represent the minimal active principle of peptidoglycan (2). Besides its capacity to potentiate the immunogenicity of vaccines, MDP contributes to stimulation of host defense as well as resistance to cancer (3). Furthermore, MDP has been shown to prime for increased responsiveness toward LPS in vitro and in vivo (4–11). This priming effect is of great importance because our understanding of pattern recognition by the innate immune system so far was primarily based on studies of effects of single components such as LPS. A major obstacle in studying interactions between different bacterial cell wall components like LPS, peptidoglycan, or lipopeptides is the difficulty of purifying these components free of LPS or lipopeptide contaminations. Such purity is difficult to achieve with conventional isolation procedures: the microheterogeneity of these structures adds to the problem. For example, macrophages are activated by picogram/ml concentrations of LPS, whereas microgram/ml concentrations of most muropeptides are required to induce cytokine release.

This problem has been overcome by chemical synthesis of defined structures. Such investigations started three decades ago in the case of muropeptides with the focus on adjuvant activity (12–16). At this time, pyrogen exclusion by the Limulus amebocyte lysate (LAL) assay was in its infancy. More recent work addresses the anti-tumor activity of synthesized conjugates of MDP (17) or the ability of glycoside derivatives of MDP to release IL-1β or TNFα from murine macrophages (18). However, no pyrogen exclusion was performed in these studies. It thus appeared timely to readdress the structure-function relationship of muropeptide activity in the light of the emerging knowledge on bacterial pattern recognition. We therefore complemented a panel of commercially available muropeptide derivatives with nine structures that we synthesized ourselves. Four of these structures were synthesized for the first time. The new structures included typical peptidoglycan breakdown products of the Gram-negative and Gram-positive cell wall, like M(AoQ) (usually ill-defined as MDP), M(A), which carries only one amino acid, and A,M, which consists of muramic acid (M) in an anhydro form, which are released during bacterial growth during infection or by bacteriolysis resulting from host defense mechanisms or administration of antibiotics (19, 20). Furthermore, we synthesized derivatives containing diaminopimelic acid for the first time. These substances underwent a thorough chemical analysis by NMR and MS as well as LAL testing. These highly defined muropeptide structures representing natural breakdown products of peptidoglycan of Gram-negative and Gram-positive bacteria, as well as some variants that are not found in nature, were tested as to their ability to synergize with LPS in inducing cytokine release.

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The abbreviations used are: MDP, muramyl dipeptide; LPS, lipopolysaccharide; LAL, Limulus amebocyte lysate; IL, interleukin; TNF, tumor necrosis factor; m-DAP, meso-diaminopimelic acid; Dpm, diaminopimelic acid; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
**Structural Requirements of Muropeptides to Synergize with LPS**

**TABLE I**

| No. | Abbreviation | Nomenclature | Source |
|-----|--------------|--------------|--------|
| 1   | M            | MurNAc       | Sigma  |
| 2   | αM           | 1,6-Anhydro-MurNAc | NK-β- |
| 3   | M(αA)        | MurNAc       | NK-α   |
| 4   | M(αAni(Q))   | MurNAc-α-La-t-isoGln | NK-α |
| 5   | M(αAni(Q))   | MurNAc-α-La-t-isoGln | Bachem-α |
| 6   | M(αA(Q))     | MurNAc-α-La-t-isoGln | Bachem |
| 7   | M(αAni(Q))   | MurNAc-α-La-t-isoGln | Bachem |
| 8   | M(αAni(αE))  | MurNAc-α-La-t-γ-D-Glu | Bachem-α |
| 9   | M(αAni(αEK)) | MurNAc-α-La-t-γ-D-Glu | Lys-α |
| 10  | M(αAni(αEm(αDpm)) | MurNAc-α-La-t-γ-D-Glu-meso-Dpm | NK-α |
| 11  | M(αAni(αEm(αDpm))A | MurNAc-α-La-t-γ-D-Glu-meso-Dpm-p-tα | NK-α |
| 12  | M(αA(αE-Dpm)) | 1,6-Anhydro-MurNAc-α-La-γ-D-Glu | NK-α |
| 13  | C₃pM(αAni(Q)) | 6-O-Stearyloyl-MurNAc-α-La-t-isoGln | Bachem |
| 14  | M(αAni(αK(αC₃p))) | MurNAc-α-La-t-isoGln-N-c-stearyloyl-L-Lys | Sigma |
| 15  | M(αAni(Q))   | GlcNAc-bt-1-N4MurNAc-α-La-t-isoGln-α | Calzyme-α |

* a Represent structural compounds of peptidoglycan.

**MATERIALS AND METHODS**

**Muropeptides**—Our strategy to synthesize several natural and non-natural muropeptides (Table I, compounds 2–4 and 8–13) was partly based on the previously published synthesis of differentially protected meso-diaminopimelic acid (m-DAP) (21, 22). To this end bifunctional DAP (Table II, a) was synthesized from benzylxycarbonyl-protected i-glutamate and a known glycine phosphate (23, 24) via a Wittig–Horner reaction to get the 2,3-dehydro-DAP. Catalytic hydrogenation using Wilkinson’s catalyst gave a 3:2 tv. ratio of the newly created stereogenic center. Final separation by medium pressure liquid chromatography yielded both L, and D,meso-diasteromers. The assignment of the stereogenic centers of the DAP dimethylster was accomplished by conversion of each single diastereomer to the bis-N-carbamoyl amide derivative and NMR analysis (600 MHz) of the methyl ester region [25]. The muramic acid moiety (Table II, b) was synthesized according to our previously published method (26, 27) from 1,3,4,6-tetra-O-acetyl-2-azido-2-deoxyglucopyranose (28). Saponification of the methyl ester of two and attachment of the first amino acid (L-alanine methyl ester) prevented the often observed formation of muramic acid 1,2-lactam during azide reduction (29, 30). The resulting sugar building block (Table II, c) was used for the synthesis of larger muropeptides. For 1,6-anhydromuramic acid, a new and efficient synthetic route was established: 4-

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**Whole Blood Incubations**—Incubation of human whole blood in the presence of the bacterial stimuli was performed essentially as described [36]. Heparinized blood freshly taken from healthy volunteers was diluted 5-fold with RPMI 1640 (BioWhittaker). The different stimuli were prepared as stock solutions in aqua ad injectabile (Braun, Mel sungen, Germany). LPS from *Salmonella abortus equi* was purchased from Sigma and either used directly or after a further purification to eliminate putative lipopeptide contaminations by a phenol re-extraction according to Hirschfeld et al. [37]. Incubations were carried out in open polypropylene reaction tubes (Eppendorf, Hamburg, Germany) at 37 °C and 5% CO₂ for 24 h. Cell-free supernatants were obtained by centrifugation at 400 × g for 2 min (Heraeus, Hanau, Germany) and stored at −80 °C until measurement.

**Monocytes**—Peripheral blood mononuclear cells of healthy volunteers were prepared with CPT™ Cell Preparation Tubes (BD Biosciences). Negative isolation of monocytes was done with the Monocyte Isolation kit (Miltenyi, Bergisch Gladbach, Germany) according to the manufacturer’s protocol. Cells were plated in 96-well plates at a density of 5 × 10⁶ cells/well in RPMI in the presence of 10% fetal calf serum (Biochrom, Berlin, Germany) and stimulated for 24 h at 5% CO₂ and 37 °C. Supernatants were stored at −80 °C until measurement.

**Isolated Liver Perfusion**—C57BL/6 mice (Charles River, Sulzfeld, Germany, bred in the internal animal facility of the University of Konstanz) were pretreated (intraperitoneally) for 1 h with 2.5 µg/kg LPS, 700 µg/kg galactosamine (GalN, Roth, Karlsruhe, Germany), and 250 µg/kg M(ADiQ). After 1 h mice were put to sleep intravenously with 150 mg/kg pentobarbital (Narcorex®, Merial, Halbergmoos, Germany) and 0.8 mg/kg heparin (Sigma). The vena portae and vena cava inferior of the mouse liver (C57BL/6) were cannulated and the organ was perfused blood-free with a modified Krebs-Henseleit buffer with a total volume of 25 ml of buffer in closed recirculation under constant pressure conditions and samples were obtained for the times indicated in the graph. The temperature of the perfusate was kept constant at 37 °C and oxygenation with pure oxygen at a pressure of 500 mbar was performed. During perfusion, the perfusate flow-through of the liver as well as the pressure were constantly measured and recorded by special software (Lab View, National Instruments, Austin, TX). Samples for alanineaminotransferase and lactate dehydrogenase measurements...
primers (0.5 μM H9262 (Roche) according to the manufacturers protocol. The MgCl₂ concentration was followed by a melting program that started at 54 °C and an elongation time of 11 s for TNF-α and GAPDH products were 93 and 86.5 °C, respectively.

Cytokine Determination—Cytokines were measured by ELISA based on antibody pairs from Endogen, Biozol, Eching, Germany (TNFα and IL-1), R&D Systems, Minneapolis, MN (IL-6), and BD Pharmingen (IL-10 and interferon γ). Recombinant cytokines serving as standards were from National Institute for Biological Standards and Control (Hertfordshire, United Kingdom) (TNFα and IL-1), from Thomae (Biberach, Germany) (interferon γ), and BD Pharmingen (IL-6 and IL-10). Binding of biotinylated antibody was quantified using streptavidin peroxidase (Dianova, Hamburg, Germany) and the substrate TMB (3,3′,5,5′-tetramethylbenzidine, Sigma).

Preparation of mRNA and Reverse Transcription—For preparation of mRNA, human whole blood incubations were stimulated with 100 pg/ml LPS and 20 nM M(ADiQ) for 2 or 4 h. After 2 h, 1 μM actinomycin D was added to some incubations and incubation was continued for a further 2 h. mRNA was isolated with the QiAmp RNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturers instructions. Possible contaminating DNA was digested with the RNase-free DNase set (Qiagen). Reverse transcription for PCR was performed using PerkinElmer Life Sciences GeneAmp PCR system 9600 with 5 mM MgCl₂, 2 μl of 10× PCR-buffer II, 1 mM dNTP (Roche Diagnostics), 2.5 μM oligodT18 (Thermo Hybird, Ulm, Germany), 20 units/μl RNAse inhibitor (Roche), 50 units/μl murine leukemia virus-reverse transcriptase (PerkinElmer Life Sciences), and 4.2 μl of RNase-free water.

Real-time PCR—Real-time PCR was performed on a LightCycler rapid thermal cycler system (Roche). The cDNA for TNFα and GAPDH was amplified using LightCycler FastStart DNA Master SYBR Green (Roche) according to the manufacturers protocol. The MgCl₂ concentration was adjusted to 3 mM TNFα and 4 mM GAPDH. The thermal cycling was performed (50 cycles) with an annealing temperature of 65 °C and an elongation time of 11 s for TNFα and 18 s for GAPDH. The amplification was followed by a melting program that started at 54 °C for 45 s and then increased to 95 °C at 0.1 °C/s. The specific melting temperatures for TNFα and GAPDH products were 93 and 86.5 °C. All primers (0.5 μM) were from Thermo Hybrid: TNFα, 5′-GAGTGCAAG-GCCCTGTTAGCCATGTGAGCAGA-3′ (forward), 5′-GCAATGATCCCA-AAGTAGACCTGCCCAGACT-3′ (reverse); GAPDH, 5′-GAAGGTGAA-GGAAGATGGTGATGGGATTTC-3′ (forward), 5′-GAAGATGGTGATGGGATTTC-3′ (reverse). The amplification products had a length of 226 (GAPDH) and 249 bp (TNFα), respectively.

Statistics—Statistical analysis was performed using the GraphPad Prism program (GraphPad Software, San Diego, CA). All data are mean ± S.E. Significance of differences was assessed by the Wilcoxon test for two groups and by analysis of variance employing the Bonferroni test for experiments with more than two groups. Non-linear regression was calculated according to the application of the Gompertz equation for the study of Xylem cell development by Rossi et al. (46). In the figures *, **, and *** represent p values <0.05, <0.01, and <0.001, respectively.

RESULTS

Synergistic Effect of LPS and M(AniQ)—To determine the most pronounced synergistic effect of LPS and M(AniQ), human whole blood was incubated with 100 pg/ml LPS and increasing amounts of M(AniQ) (2 pg to 200 ng/ml). LAL-negative muropeptides alone induced essentially no cytokine release (<16 pg of TNFα/ml of blood). A significant synergistic effect between LPS and M(AniQ) was observed from a concentration of 20 nM M(AniQ), which corresponds to 10 ng/ml M(AniQ) (Fig. 1). Because commercially available LPS preparations might contain highly bioactive contaminants that can effect biological read-outs, we further purified the LPS by phenol re-extraction as published by Hirschfeld et al. (37). However, as shown in Fig. 2, the capacity of LPS to induce cytokine release from human whole blood was not significantly altered by the re-purification procedure. Furthermore, the synergistic effect of M(AniQ) was not dependent on the purity of the LPS preparation.

To investigate the effect of M(AniQ) preincubation, human whole blood was incubated with 20 nM M(AniQ) for 4 h and re-stimulated with 100 pg/ml LPS. The results were compared with an incubation in which LPS and M(AniQ) were added simultaneously. Preincubation of M(AniQ) led to a 10% increase of TNFα release compared with simultaneous stimulation with LPS (data not shown). Because this difference was not statistically significant, stimuli were added together in all further experiments.

As indicated in Fig. 3a, costimulation of human whole blood with different concentrations of LPS and 20 nM M(AniQ) led to a shift to the left of the concentration-response curve of LPS-induced TNFα release by about 3 log orders. Similarly, in isolated monocytes in the presence of 20 nM M(AniQ), 100 pg/ml
LPS achieved TNFα levels comparable with stimulation with 1 μg/ml LPS (Fig. 4). Furthermore, the synergistic effect of LPS and M(AniQ), although to a lesser extent, was also observed for the release of IL-1β, IL-6, and IL-10 (Fig. 3, b and c). In extension, M(AniQ) significantly enhanced the LPS-induced liver injury in galactosamine-sensitized mice, measured in situ as alanine aminotransferase liberation into the perfusate of isolated livers (Fig. 5), indicating that ex vivo a similar synergistic endotoxic liver injury is observed such as in vivo.

Kinetics of TNFα Release and mRNA Formation in the Presence of M(AniQ)—TNFα release from human whole blood was determined at different time points after stimulation with 100 pg/ml LPS alone or costimulation with 20 nm M(AniQ). A synergistic effect was evident after 4 h of incubation. Maximal TNFα release was reached after 8 h in both series of experiments, indicating that M(AniQ) did not alter the kinetics of TNFα release (Fig. 6).

To investigate whether the synergistic effect of M(AniQ) on LPS-induced TNFα release is because of enhanced transcription of TNFα mRNA, human whole blood was stimulated with 100 pg/ml LPS ± 20 nm M(AniQ) in the presence or absence of 1 μM actinomycin D. TNFα mRNA was determined by quantitative real-time PCR. Samples costimulated with LPS and M(AniQ) showed an increased expression of TNFα mRNA after 4 h compared with stimulation using LPS only. The presence of 1 μM actinomycin D blunted the effect of M(AniQ) on TNFα mRNA expression, whereas GAPDH levels remained unchanged. These results indicate that M(AniQ) increases LPS-induced TNFα mRNA, which seems to be because of increased de novo transcription rather than to an increase in mRNA stability (Fig. 7).

Dependence of Synergy on Muropeptide Structure—Fifteen muropeptides with different structures were investigated with regard to their synergistic activity with LPS in monocyte activation. Nine of the different muropeptide derivatives, which were not commercially available, were synthesized, the others were bought in highly pure quality (Table I). Purity and identity was confirmed by LAL, NMR, and MS analysis for all muropeptides. Control experiments confirmed that 20 nm of all muropeptides induced no significant cytokine release from human whole blood. The muramic acid (M) and the anhydroform of the muramic acid (AM) showed no synergistic effect when coinoculated with LPS (data not shown). Furthermore, as depicted in Fig. 8, the muropeptide M(A), which carries only the amino acid L-alanine, also did not synergize with LPS, but a strong synergism was observed if this structure was extended by the addition of the amino acid isoglutamine, i.e., M(AiQ). M(AniQ), with the natural N-acetylglucosamine (GM(AniQ)) showed a similar synergistic effect.

The peptidoglycans of different bacteria have similar structures although the amino acids of the peptide subunits differ. Therefore, we synthesized M(AniQ), changed the isoglutamine to a glutamate (M(AniQE)), and additionally a derivative extended by l-lysine (M(AniQL)). Both changes did not result in loss of synergistic activity compared with M(AniQ). However, when the third amino acid was replaced with diaminopimelic acid (M(AniQEDpm)) the synergistic effect was completely abolished. Synergism could also not be restored by addition of alanine (M(AniQEDpmN1)). Derivatives, carrying an anhydro-muramic acid and diaminopimelic acid in either the meso (M(AniQEDpm) or LL form (M(AniQEDpm)) were also inactive.

The amino acids alanine and isoglutamine in the peptide string of M(AniQ) are naturally found in the LD conformation, according to Fischer's nomenclature. When the stereochemical formation was changed to the LL or DD forms (M(AniQ) and M(AniQ), respectively, no synergistic effect could be observed, indicating that the recognition is stereoselective. This series of molecules shows that M(AniQ) and (M(AniQE) represent the
minimal synergistic structure of muropeptides and that exactly two amino acids are required to reach the maximal synergistic effect.

Artificial lipophilic muropeptides have the advantage of being less rapidly eliminated in vivo. We found that M(AniQ) carrying an artificial fatty acid ester bound to M(C18M(AniQ)) showed a synergistic effect comparable with M(AniQ), whereas the addition of an artificial fatty acid ester bound to the peptide chain (M(AniQKEC18)) reduced the synergistic effect (Fig. 8).

**Possible Antagonistic Effects of Small Muropeptides**—To investigate whether the synergistic effect of M(AniQ) and LPS can be antagonized by smaller muropeptides, human whole blood was stimulated with 100 pg/ml LPS plus 20 nM M(AniQ) in the presence or absence of 20 nM M(AniQ). TNFα (a), IL-6 (b), and IL-10 (c) levels were determined by ELISA after 24 h of incubation in the cell-free supernatants. Non-linear regression and EC50 were calculated according to Gompertz. Data are mean ± S.E.

and M(AniQ) was not significantly reduced (maximally ~25%) in any experiment indicating that the synergistic effect of M(AniQ) is not antagonized by muropeptide structures smaller than the M(AniQ).

**DISCUSSION**

The adjuvant activity of peptidoglycan has been attributed to muropeptide structures, the dipeptide M(AniQ) (commonly but ill defined abbreviation MDP) being the minimal structure (2). The synergy of LPS and muropeptides to induce cytokines represents a likely but yet unproven explanation for the adjuvant activity of muropeptides. An in vitro synergism of M(AniQ) with LPS in inducing cytokines was shown in various experimental settings including murine macrophages, human monocytoic cell lines, and whole blood (4, 6, 8, 10). M(AniQ) alone has several times been reported to be capable of stimulating cytokine release (12, 14, 18, 39–41). However, in all these studies very high concentrations of M(AniQ) (i.e. 1 µg/ml up to 100 µg/ml) were used and in most cases no exclusion of endotoxin contamination was carried out. Here, we demonstrate that in human whole blood as little as 20 nM (i.e. 10 ng/ml) M(AniQ) and M(AniQ) derivatives of highly pure quality sufficed to increase the TNFα releasing capacity of LPS about 4-fold and induced a shift to the left of the concentration-response curve by a factor of 1000. In our hands, none of the LAL negative preparations showed any cytokine inducing activity alone. Furthermore, this study provides a detailed biochemical analysis of the structural requirements of muropeptides to synergize with LPS in a physiological environment.

M(AniQ), because of its low molecular weight and water solubility, is rapidly excreted from the body. After intravenous injection of M(AniQ) (1.5 mg/kg) in rats, M(AniQ) plasma levels immediately start to decline. Thirty minutes after injection the M(AniQ) plasma level went from 27 to 4 ng/ml and was no longer detectable after 2 h (42). Microgram/ml quantities of muropeptides therefore might be relevant in case of adjuvant activities for vaccination purposes but do not seem to reflect the conditions during bacterial infection.

The very low levels of muropeptides, i.e. 10 ng/ml in case of M(AniQ), which are shown here for the first time, to suffice to synergize with low quantities of LPS, represent an observation of major importance. The fact that the synergism is also seen with highly purified LPS indicates that no contaminants like lipoproteins are responsible for this effect. Furthermore, the experiment with isolated monocytes shows that the synergistic effect is not dependent on a bystander effect of further blood cells or serum components. The observed synergism was most
pronounced for the release of TNFα, but translated also to other cytokines such as IL-1β, IL-6, and IL-10, indicating that the effect is more general. Wang et al. (10) reported a 3-fold increase of LPS (10 ng/ml)-induced TNFα from human whole blood when coincubated with 1 μg/ml M(AniQ) and a 2-fold increase in IL-6 release, whereas IL-10 remained unchanged. The difference in IL-10 release might be because of the use of different stimulus concentrations as well as differences in the incubation time (6 h in the study of Wang et al. (10) versus 24 h in our study). IL-10 is produced late after the initial activation of monocytes and not released in major amounts before 8 h (data not shown).

The addition of M(AniQ) enhanced LPS-induced transcription of the TNFα mRNA and expression of the TNFα protein, but did not alter the kinetics of TNFα release. This is in line with the findings of Wolfert et al. (4) who reported an increased transcription of LPS-induced TNFα mRNA in MonoMac 6 cells when coincubated with 100 μg/ml M(AniQ). However, in their experimental setting, 100 μg/ml M(AniQ) alone induced a 10-fold induction of TNFα mRNA, which did not result in protein expression. The lowest M(AniQ) concentration investigated in their study was 10 ng/ml, which, however, did not induce TNFα mRNA (4). Unfortunately, this concentration was not tested for a putative synergism with LPS.

Furthermore, the results of our study indicate that the synergistic effect of LPS and M(AniQ) also translates to mature macrophages in their organotypic environment. In situ perfusion of livers from mice isolated after in vivo treatment showed a significantly greater damage when coincubated with LPS and M(AniQ), compared with LPS alone. Synergy with regard to lethal toxicity in vivo has been reported earlier (9, 43, 44). The results of our ex vivo experiment show that the synergy takes place at the level of injury of target organs.
now, a large number of synthetic muropeptide derivatives have been developed for vaccination purposes and their biological activity has been intensively characterized (1, 13). Among these adjuvants were hydrophobic acyl derivatives, derivatives with chemical modifications at the carboxyl group, and several others. However, none of these structures is of relevance during natural bacterial infections. Kotani et al. (15) reviewed the immunopharmacological activities of M(AiniQ) and its analogs. In line with our findings they reported that M(AiniQ) and M(AyoE) were most potent, whereas the corresponding stereoisomers (LL or DD configuration) were inactive. However, no synergistic effects with LPS in the case of cytokine release were addressed in this review. The stereoselectivity of the adjuvant and toxic action of muropeptides has been demonstrated (14, 44, 45). Our finding that the addition of a C18 st M does not alter the synergistic qualities, whereas comparable modification at the peptide chain blunted the effect further pronounces the importance of the amino acids and their configuration.

The introduction of Dpm into the muropeptide led to a reduction of the synergistic effect. Not much is known about the synergistic action of muropeptides containing Dpm. Takada et al. (44) showed that the M-tripeptide (M(AyoQDpm) prepared from Lactobacillus plantarum containing Dpm primes for anaphylactoid reactions induced by LPS in C3H/HeJ mice, but its priming activity was weak compared with M(AiniQ). Furthermore, it remains to be defined whether the nature of synergism described in these experiments in LPS non-responsive mice is translatable to the synergism described in LPS responsive cells. GmM(AyiQMaeDpmnA) was reported to stimulate cytokine expression from human monocytes and GmM(AyoEmDpmA) was shown to synergize with LPS in the activation of hamster epithelial cells (5, 40). Both substances contained Dpm. However, the structures although quite similar to our M(AyiEmDpm) or M(AyoEmDpmA) are not the same and furthermore, have been obtained by biochemical digestion and not by synthesis. Because microgram/ml quantities are used, neither contaminating endotoxins nor further muropeptides with a higher biological activity can be fully excluded although LAL testing was performed.

So far, no study has been shown employing synthetic muropeptides larger than a dipetide at relevant concentrations, and that such molecules have any biological activity. A proportion of 1% M(AiniQ) and less when employing microgram quantities cannot be excluded.

Taken together, our data suggest that muropeptides are natural amplifiers of biological effects. The responses to LPS differ dramatically in the absence or presence of muropeptides from Gram-negative and Gram-positive cell walls, which generally accompany LPS in natural infections. The finding that the LAL-negative preparations used here did not induce any cytokine release in human whole blood even at microgram/ml concentrations characterizes the muropeptides rather as synergists, than as independent immune stimuli. Therefore, antagonizing such effects might represent an interesting anti-inflammatory strategy leaving the LPS-based recognition of pathogens intact.