Chapter 8

The Naturally Occurring Muramylpeptide G(Anh)MTetra Protects Mice against *Escherichia coli* Septic Shock and Induces Neutrophilia *in vivo*

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Summary

Several constituents of the bacterial cell are recognized by the immune system and induce an inflammatory response. Especially components of the bacterial envelope are potent inducers of host defense mechanisms. The liberation of these components during the course of an infection can result in an overstimulation of the immune system. This, in turn, may even lead to septic shock, a very severe clinical condition with a high mortality rate. As the lipopolysaccharide (LPS) of gram-negative bacteria is one of the most powerful immunostimulatory agents known, the effects of this molecule have been extensively studied. However, the contribution to the immune response of other bacterial inducers, such as peptidoglycan, has received less attention. Most studies on the immunostimulatory effects of peptidoglycan have been performed with gram-positive peptidoglycan or synthetic analogs of peptidoglycan fragments, such as muramyldipeptide (MDP). To assess the possible role of peptidoglycan in gram-negative infections, this study focuses on the effects of *N*-acetylglucosaminyl-1,6-anhydro-*N*-acetylmuramyl-L-alanyl-D-glutaminyl-m-diaminopimelyl-D-alanine (G(Anh)MTetra), a peptidoglycan degradation product from gram-negative bacteria. Based on the ability of this muramylpeptide to induce the expression of inflammatory cytokines *in vitro*, its *in vivo* effects were studied in an animal model of gram-negative septic shock. It could be shown that treatment with small amounts of G(Anh)MTetra protected mice against *E. coli* induced lethality. Furthermore, G(Anh)MTetra was tested in immunocompromised mice. The increase in the number of neutrophils, which are very important cells in the defense against microorganisms, could be significantly enhanced by treatment with G(Anh)MTetra.

Introduction

During a bacterial infection it is of vital importance to the infected host that the presence of pathogenic bacteria is detected as soon as possible in order to mount an effective counterattack. It is therefore not surprising that bacterial constituents that are present on the outside of the cell and therefore readily accessible to detection, have been selected by the immune system as indicators of bacterial presence and potent inducers of a host-response. Of all the different surface constituents of bacteria, most attention has been focused on the lipopolysaccharide (LPS) of the gram-negative outer membrane as an inducer of host defense mechanisms. Extensive studies on the immunostimulatory properties of LPS showed that the molecule is a potent inducer of the inflammatory cytokine cascade and as such is an important effector molecule in bacteremia and septic shock (5). In particular the release of tumor necrosis factor (TNF) by cells of the monocyte-macrophage lineage upon stimulation with LPS pointed to this molecule as being the major culprit in septic shock and indeed a septic shock like pathology can be induced with purified LPS in animal models (5, 17, 32). Based on these observations, several anti-LPS

approaches have been developed and clinically evaluated for the treatment of septic shock in recent years (36). However, it became apparent that these approaches tackled the sequence of events leading to septic shock at a much too early stage. This stage has been long passed when the syndrome is diagnosed and as a result the clinical efficacy of such approaches proved to be very disappointing, as is best illustrated by centoxin, a monoclonal antibody directed against the lipid A moiety of LPS, the clinical failure of which aroused a lot of attention (36). The research focus therefore has shifted to later events and modulation of the responsible mediators of the immune system is currently attempted. These approaches include the use of TNF receptor fusion proteins (46) or monoclonal antibodies directed against TNF.

Gram-negative bacteria are the major causes of septic shock in the hospital setting (around 60% of all cases). A possible explanation for this may be that LPS as a molecule derived from gram-negative bacteria only, is such a very potent inducer of inflammation. However, gram-positive bacteria, the cell-wall of which lacks LPS, can cause the same pathophysiology. This implies that other bacterial elicitors must also be able to induce the immune system in a similar way. As in gram-positives the peptidoglycan is the major constituent of the cell wall and is located on the outside of the cell, this macromolecule makes a good candidate for such and elicitor.

The peptidoglycan is a heteropolymer, composed of glycan-strands crosslinked by peptides, that completely encloses that cell. It is responsible for the structural integrity of the cell wall. The adjuvant properties of peptidoglycan have long been recognized. Freund prepared a suspension of heat-killed mycobacteria in oil in his famous adjuvant formulation (19). The active component was later shown to be peptidoglycan. An approach to identify the smallest peptidoglycan fragment that still showed adjuvant activity, resulted in the synthesis of the peptidoglycan substructure muramyldipeptide (MDP) (15). Hundreds of derivatives of MDP have since then been synthesized and tested for adjuvant activity, and some of these are clinically used as adjuvants and in anti-cancer therapy (1, 2). Although MDP mimics many of the biological effects of peptidoglycan and displays the ability to induce an inflammatory host response, one has to bear in mind that it is an synthetic derivative and neither is released by bacteria *in vivo* nor is produced by phagocytes after ingestion of bacteria (47). Therefore its value in studying the contribution of naturally occurring bacterial peptidoglycan fragments to the pathophysiology of septic shock is limited.

The role of peptidoglycan and that of other effector molecules such as teichoic acids and exotoxins in gram-positive bacteremia has been evaluated by several research groups (22, 24, 31, 45). However, the possibility that peptidoglycan may also play a role in gram-negative sepsis is usually overshadowed by the attention given to LPS. Even though the peptidoglycan in gram-negatives is not directly accessible due to the presence of the outer membrane, peptidoglycan fragments are shed by several bacteria during growth and are released in large amounts upon (antibiotic induced) lysis. Therefore it could well be that peptidoglycan shows synergy with LPS in induction of the host-response. As the major peptidoglycan fragments that are released by gram-negative bacteria are the 1,6-anhydromuropeptides, we decided to focus on these naturally occurring muramylpeptides and study the potential contribution of these to the inflammatory response. These 1,6-anhydromuropeptides are released by the gram-negative pathogens *Neisseria gonorrhoeae* and *Bordetella pertussis* during normal growth and have been proposed to function as virulence factors for these bacteria (7, 28, 37, 40). The enzymes that are responsible for the

production of these muropeptides are the lytic transglycosylases, a class of bacterial peptidoglycan hydrolases of which members have been identified in several gram-negative bacteria and that also seems to be responsible for the massive degradation of peptidoglycan during lysis (8-10, 25). One of these enzymes, the soluble lytic transglycosylase (Slt70) from *Escherichia coli,* was used for the large scale production of 1,6-anhydromuropeptides (16). In earlier studies we showed that these muropeptides are potent inducers of IL-1, IL-6 and G-CSF in human monocytes (11, 12). In this study, we set out to assess the *in vivo* activities of these muropeptides, focusing on their effects in a model of *E. coli* induced septic shock and their potency to restore neutrophil counts in neutropenic mice.

Materials and Methods

Mice

Female Swiss albino and BALB/c mice that were 6 to 8 weeks old, weighing 18-20 gram, were obtained from BRL (Füllinsdorf, Germany).

Bacterial preparations and counts

E. coli ATCC 25922 was obtained from the American Type Culture Collection. The strain was passaged in mice 6 times to enhance virulence, resulting in a LD50 of 4x 10^4. Bacteria were cultured in Luria Bertani (LB) medium and dilutions of fresh overnight cultures were used for infection. Bacterial counts were determined by the inoculation of serial dilutions on LB-agar plates.

Reagents

MDP (*N*-acetylmuramyl-L-Ala-D-isoGln), *E. coli* lipopolysaccharide serotype 055:B5 and cyclophosphamid were purchased from Sigma Chemical Co. (St. Louis, USA) and were dissolved or suspended in phosphate-buffered saline (PBS). Recombinant human G-CSF (Neupogen) and Ceftriaxone (Rocephin) were from Hoffmann-La Roche (Basel, Switzerland). G-CSF was dissolved in PBS+0.2% gelatin. The TNF-receptor-IgG1 fusion protein was obtained from Dr. Lesslauer, Hoffmann-La Roche, Basel and was dissolved in 0.9% saline (46). All reagents were tested for LPS contamination using the *Limulus* amebocyte lysate detection (LAL) system and were found to contain <0.05 EU/ml (< 5 pg endotoxin/ml). All other chemicals used were of the highest grade commercially available.

Purification of G(Anh)MTetra

Sacculi were isolated from *E. coli* cells as described (21) and were degraded batchwise with purified soluble lytic transglycosylase (Slt) in 20 mM phosphate buffer pH 6.8, using 120 µg Slt per mg of sacculi. After overnight incubation at 37°C, Slt was inactivated by boiling for 5 minutes and the denatured protein was removed by centrifugation. Aliquots of the supernatant were applied to an HPLC system, optimized for the separation of 1,6-anhydromuropeptides as described (8). Fractions containing individual muropeptides were collected and pooled fractions were lyophilyzed. Purity of the preparations was determined by HPLC and the identity of the muropeptides was confirmed by electron spray ionization tandem mass spectroscopy (EI-MSMS). The muropeptides were dissolved in PBS and the

concentration was determined by amino acid analysis. The LPS content was determined using the LAL-assay.

Neutropenia model

The mice were made neutropenic by intraperitoneal (i. p.) injection of 150 mg / kg cyclophosphamid (CPA). Recovery from neutropenia was studied in 6 groups of 24 mice (A-F). Group A served as a non-neutropenic negative control and was only treated with vehicle (PBS). Group B-F were injected subcutaneously with the following modulators for five consecutive days after CPA treatment: vehicle (B), 2.5 µg G-CSF (C), 1.67 µg G(Anh)MTetra (D), 0.97 µg MDP (E) and 2.5 µg LPS (F). Heart blood was taken from 3 animals per group per day, starting on the first day after CPA treatment and neutrophil counts were determined in duplicate by Pappenheim staining of blood smears.

E. coli shock model

E. coli septic shock was induced in mice by intraperitoneal (i. p.) injection of 0.5 ml of a 500 fold diluted *E. coli* ATCC 25977 culture, corresponding to 10^6 colony forming units. After 4 hours the infection was stopped by subcutaneous injection of 1 mg / kg ceftriaxone. In a typical experiment the mice died 24-48 hours after infection and were sterile at the time of death. Survival was monitored daily for one week. Animals surviving after this period remained alive.
The protective effect of G(Anh)MTetra in comparison with that of MDP was studied by pretreatment of the mice, according to the following administration schedule. Groups of 10 mice (A-E) were pretreated by subcutaneous injection (0.2 ml) of vehicle (A), 0.05 µg G(Anh)MTetra (B), 5 µg of G(Anh)MTetra (C), 2.7 µg MDP (D) and 2 µg G-CSF (E) 48 hours before infection. This treatment was repeated 24 hours and 2 hours before infection. The last pretreatment of group E with G-CSF was combined with an i.p. administration of 50 µg of TNFR-IgG1.

Results

G(Anh)MTetra purification.

A total of 40 HPLC runs yielded 6 mg of the monomeric muramyltetrapeptide *N*-acetylglucosaminyl-1,6-anhydro-*N*-acetylmuramyl-L-alanyl-D-glutaminyl-m-diaminopimelyl-D-alanine (G(Anh)MTetra). The purity of the muropeptide was >95% as determined by HPLC and the LPS contamination of the preparation proved to be less then 0.12 EU / mg G(Anh)MTetra. The identity of G(Anh)MTetra could be confirmed by tandem mass spectroscopy and the structure of this muropeptide is shown in Fig. 1.

G(Anh)MTetra is protective against E. coli induced shock.

Based on the protective effect of the synthetic muropeptide MDP in different models of bacterial infection and on the observation that G(Anh)MTetra is capable of inducing G-CSF expression *in vivo*, we set out to assess the potency of G(Anh)-MTetra, as a naturally occurring muropeptide, in protection against gram-negative septic shock, and compared this proposed protection with that induced by MDP.

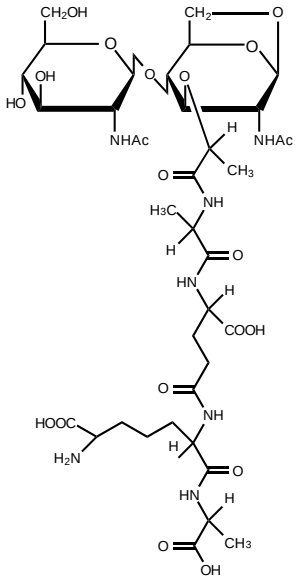

FIG1. Structure of G(Anh)MTetra (*N*-acetylglucosaminyl-1,6-anhydro-*N*-acetylmuramyl-L-alanyl-D-glutaminyl-m-diaminopimelyl-D-alanine).

Table 1. Survival rate of Swiss albino mice in the model of *E. coli* induced shock.

Group	No. of mice	Pretreatment	Percentage survival (%)*
A	10	PBS (-48, -24, -2 hr)	0
B	10	0.05 µg G(Anh)MTetra (-48, -24, -2 hr)	10
C	10	0.5 µg G(Anh)MTetra (-48, -24, -2 hr)	30
D	10	2.7 µg MDP(-48, -24, -2 hr)	0
E	10	2 µg G-CSF (-48, -24 hr) 2 µg G-CSF + 50 µg TNFR-IgG1(-2 hr)	60

* Survival percentage was recorded after 7 days

As shown in Table 1, pretreatment of mice with G(Anh)MTetra indeed shows a clear protective effect in the *E. coli* induced septic shock model. Pretreatment with 0.05 µg and 5 µg G(Anh)MTetra resulted in a survival percentage after 7 days of 10% and 30%, respectively. It should also be noted that the surviving mice were healthy looking and behaving normally. Treatment with a dose of MDP equimolar to the highest G(Anh)MTetra dose did not show any protective effect. The positive control, G-CSF plus TNFR-IgG1 resulted in 60% survival which was somewhat lower than in comparable experiments where survival rates up to 100% were demonstrated for this combination.

Treatment with G(Anh)MTetra accelerates recovery from neutropenia.

To study whether the *in vivo* effects of G(Anh)MTetra are modulated through induction of G-CSF, its potential to induce neutrophilia was studied.
In comparison with MDP, G(Anh)MTetra is more potent in accelerating the recovery of neutrophil counts in mice that were made neutropenic by CPA treatment. Figure 2 shows that on day six, the neutrophil counts of animals treated with G(Anh)MTetra are already significantly elevated as compared with that of untreated animals or animals treated with MDP. Treatment with G-CSF or LPS showed a marked effect on recovery, as was to be expected based on data from the literature.

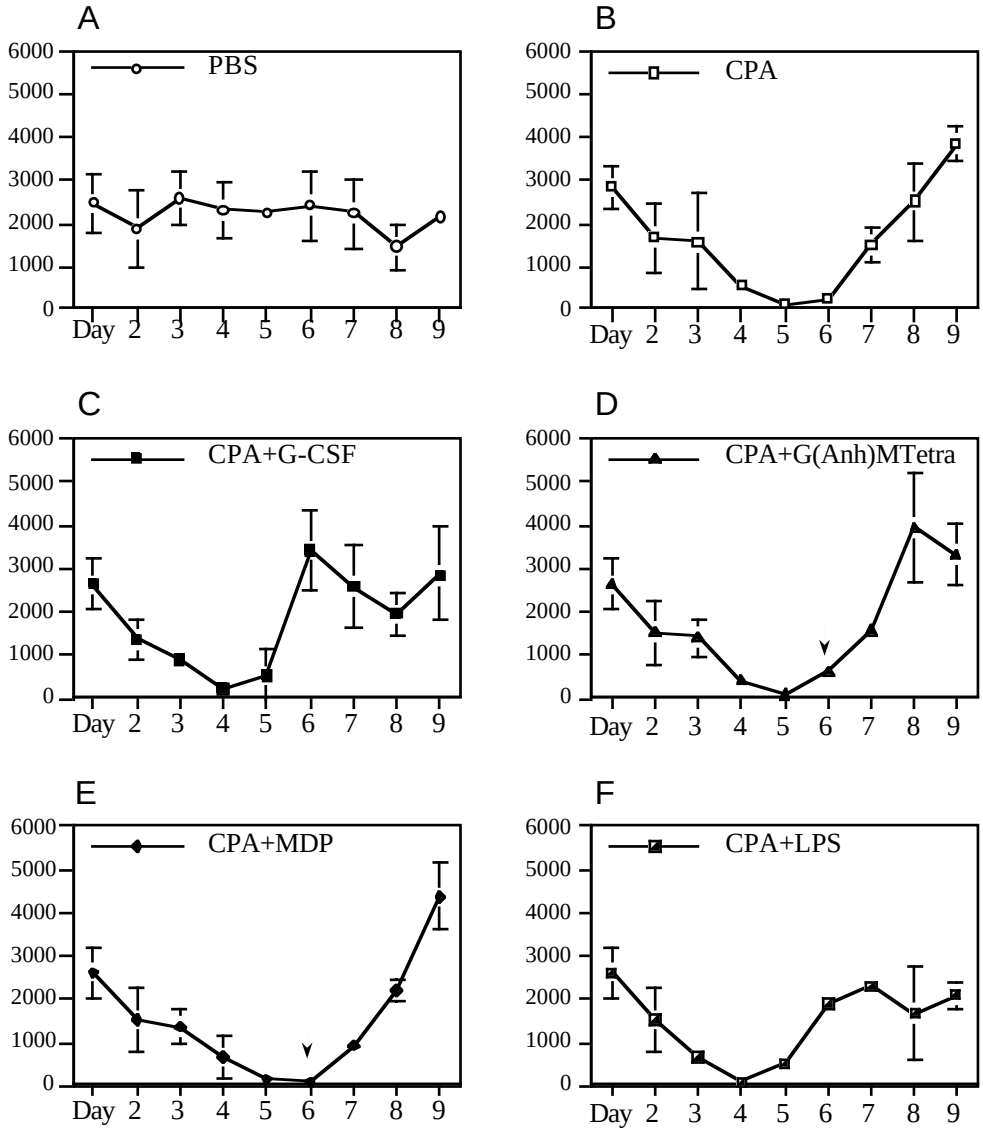

FIG 2. Comparison of the potential of different effector molecules to enhance recovery from CPA-induced neutropenia. The numbers of neutrophils per ml. of serum are displayed. The substances that were used to treat the mice are indicated on the panels. The arrows point to the most noteworthy difference between G(Anh)MTetra and MDP.

Discussion

The effects of MDP derivatives in LPS-induced models of septic shock have been extensively studied(35). Although these studies are useful for the evaluation of the possible therapeutic potential of MDP and its derivatives, they seem less suited for the elucidation of the mechanisms of gram-negative septic shock as they involve a double mimicking: first the use of LPS as the main inducer of shock and, second, the use of MDP as a mimic of the peptidoglycan fragments that are produced during a bacterial infection. For studying of the mechanisms of gram-negative septic shock and the relative contribution of the different bacterial inducers, we therefore favor the model as described in this report.

The difference in immuno stimulatory activity between G(Anh)MTetra and MDP that was observed *in vitro* (12) also holds true *in vivo* as demonstrated by the results of this study. For both the *E. coli* septic shock model and the neutropenia model small doses of G(Anh)MTetra proved to be more biologically active than equimolar amounts of MDP.

The observation that G(Anh)MTetra, although being an inducer of inflammatory cytokines *in vitro*, shows a protective effect in the *E. coli* shock model is surprising. In contrast, MDP has been shown to increase the toxic lethality of LPS in LPS-induced models of septic shock. Furthermore, pretreatment with MDP *in vitro* primes macrophages for LPS-induced cytokine gene expression (27). A recent study, however, demonstrated that different MDP-derivatives showed effects on LPS lethality that ranged from sensitization to desensitization, and which did not seem to correlate with the ability of the compounds to induce the production of inflammatory cytokines (35). It therefore seems that the inflammatory response to muropeptides involves a delicate balance that can result in either sensitization or desensitization, depending on the structure, and probably also the dose, of the muropeptides. The mechanisms that are involved this balance are not yet understood.

MDP proved to be ineffective in the *E. coli* induced septic shock model even though MDP and MDP-derivatives have been shown to enhance the non-specific resistance in several models of infection (2, 6, 29, 30). An explanation for this observation may be that the model that was used in the current study mimics the pathophysiology of gram-negative septic shock rather than infection (the animals are sterile at the time of death) and also involves lower doses of muropeptides than were used in the infection models (33).

The bacterial blood counts just before antibiotic treatment did not differ between G(Anh)MTetra-treated and vehicle-treated mice (data not shown). This indicates that the protective effect of G(Anh)MTetra can not be explained by an enhancement of the nonspecific immunity (possibly mediated by G-CSF), resulting in an enhanced phagocytosis of the administered bacteria. Furthermore, in this model of *E. coli* shock, G-CSF alone is ineffective and only shows a therapeutic effect in combination with an anti-inflammatory molecule, such as the TNF-receptor IgG1 fusion. Therefore the effect exerted by G(Anh)MTetra most likely is an anti-inflammatory one. It has been described that, apart from being inducers of the inflammatory cytokines, MDP derivatives are also able to induce the production of anti-inflammatory molecules such as the IL-1 receptor antagonist and soluble TNF receptors (4). Our results suggest that *in vivo* G(Anh)MTetra is an even more potent inducer of these negative regulators of inflammation. Also the induction of G-CSF

may contribute directly to this anti-inflammatory effect as G-CSF has been described to suppress the production of TNF, the most important mediator of septic shock (20). Another mechanism involved in the anti-inflammatory effects of G(Anh)MTetra may be the downregulation of receptors. MDP derivatives have been shown to downregulate expression of the main LPS receptor, CD14 (26), that has also been described to play a role in the activation of monocytes by peptidoglycan (48). Several binding-sites for peptidoglycan and/or MDP have been described and the expression of these may be downregulated by G(Anh)MTetra in a similar way (13, 14, 39, 41, 42, 48).

The enhanced recovery from neutropenia as stimulated by the administration of G(Anh)MTetra, indicates the induction of G-CSF by this muropeptide *in vivo* as has been observed *in vitro* (11). MDP-derivatives have also been described to have beneficial effects in leukopenia and one derivative, romurtide, is even clinically used in cancer patients for this indication (3, 38, 44). Again the *in vivo* effects of G(Anh)MTetra seem to be more potent. This difference may be explained by a difference in bio-availability or half-life. It has been shown that 90% of MDP is renally excreted within 2 hours after administration (34) and it is very well possible that G(Anh)MTetra is more slowly excreted. Furthermore, muropeptides with reducing ends (such as MDP or lysozyme products) are better substrates than 1,6-anhydro muropeptides for the peptidoglycan degrading amidase that is present in serum (43).

The observed difference between the activities of MDP and G(Anh)MTetra could also be interpreted as a difference in response of the immune system towards peptidoglycan products produced by lysozyme (mimicked by MDP), as possible indicators of a controlled bacterial presence, and those produced by bacterial enzymes (such as G(Anh)MTetra) as indicators of an uncontrolled bacterial presence. As the muropeptides of the 1,6-anhydro type are liberated in large amounts upon lysis of several (if not all) gram-negative bacteria, one could imagine that the treatment of sepsis with bactericidal antibiotics my have a detrimental effect. A similar hypothesis has been discussed for LPS release but considerable controversy still surrounds this issue (23). The studies that were aimed at finding a correlation between antibiotic treatment and septic shock progression again mainly focused on LPS and LPS induced pathways. However, the effects of peptidoglycan fragments that are released during antibiotic treatment cannot be inferred from these studies, as these fragments may induce other pathways. The fact that other pathways besides those induced by LPS are important in the pathophysiology of septic shock is illustrated by the observation that LPS-insensitive mice are still sensitive to *E. coli* induced shock (18). Also our *in vitro* results indicated different pathways for stimulation of monocytes by LPS or G(Anh)MTetra. Future studies on the contribution of peptidoglycan fragments to the progression of septic shock and the effects of antibiotic treatment, await further characterization of these pathways.

The results presented here make a strong case for the contribution of 1,6-anhydromuropeptides to the pathophysiology of gram-negative sepsis. Although the administration of small doses of G(Anh)MTetra proved to be protective in the *E. coli* induced shock model, it is very likely that the massive release during gram-negative sepsis could very well be detrimental through synergism with LPS. Therefore antibacterial approaches that would control the release of these muropeptides may prove to have added value and the enzymes that are responsible

for this release, the lytic transglycosylases, may prove to be interesting targets in this respect.

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