The Origin of the Synergistic Effect of Muramyl Dipeptide with Endotoxin and Peptidoglycan*

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Although the basis for the high mortality rate for patients with mixed bacterial infections is likely to be multifactorial, there is evidence for a synergistic effect of muramyl dipeptide (MDP) with lipopolysaccharide (LPS) on the synthesis of proinflammatory cytokines by mononuclear phagocytes. In this study, co-incubation of human Mono Mac 6 cells with MDP and either LPS or peptidoglycan (PGN) resulted in an apparent synergistic effect on tumor necrosis factor-α (TNF-α) secretion. Although incubation of cells with MDP alone produced minimal TNF-α, it caused significant expression of TNF-α mRNA. These findings suggest that the majority of TNF-α mRNA induced by MDP alone is not translated into protein. Furthermore, simultaneous incubation of cells with MDP and either LPS or PGN resulted in TNF-α mRNA expression that approximated the sum of the amounts expressed in response to MDP, LPS, and PGN individually. These findings indicate that the apparent synergistic effect of MDP on TNF-α production induced by either LPS or PGN is due to removal of a block in translation of the mRNA expressed in response to MDP. In subsequent studies, the effects of MDP alone and its effect on the production of TNF-α by LPS and PGN were determined to be independent of CD14, Toll-like receptor 2, and Toll-like receptor 4. These findings indicate that MDP acts through receptor(s) other than those primarily responsible for transducing the effects of LPS and PGN. Successful treatment of patients having mixed bacterial infections is likely to require interventions that address the mechanisms involved in responses induced by a variety of bacterial cell wall components.

Bacteremia is a critical problem in intensive care units, accounting for high morbidity and mortality rates. The mortality rate associated with bacteremia exceeds 30% (1, 2). In a recent 12-year clinical study, Gram-positive and Gram-negative bacteria accounted for 46.9 and 31.5% of bacterial episodes in an intensive care unit, respectively, with Gram-positive organisms being cultured from more patients (3). Further, the incidence of combined infections increased more than 4-fold over the 12-year period and was associated with a mortality rate exceeding 55%. Based on the fact that the majority of the deleterious effects of bacteremia are caused by inflammatory responses to specific bacterial components, these findings suggest that the patient’s response to a mixture of Gram-positive and Gram-negative organisms may be heightened to the detriment of the patient.

The two most commonly studied components of Gram-positive and Gram-negative bacterial cell walls are peptidoglycan (PGN)1 and lipopolysaccharide (LPS), respectively. Although Gram-negative bacterial cell walls also contain PGN, its concentration is far greater in the walls of Gram-positive bacteria (4). Proinflammatory effects of these bacterial cell wall components occur both in vitro after treatment of mononuclear phagocytes and in vivo after exposure of whole animals, with cells and animals being more sensitive to LPS than to PGN (5).

The results of recent experimental studies provide evidence for a synergistic effect of LPS with muramyl dipeptide (MDP), the minimal structural subunit of PGN, accounting for some of its immunogenicity (6). However, the underlying mechanism of action for MDP has not been fully elucidated. For example, there are discrepancies regarding the involvement of specific receptors, with some investigators indicating that MDP exerts its synergistic effect in human leukocytes in a CD14-dependent manner (7). Others indicate that the response is CD14- and Toll-like receptor 4 (TLR4)-independent in human monocyte cell lines and that MDP up-regulates expression of one of the primary components (MyD88 mRNA) in the TLR-mediated response to LPS (4).

We report here that MDP not only synergizes with LPS but also acts similarly with PGN to induce the synthesis of tumor necrosis factor (TNF)-α in the human monocytic cell line Mono Mac 6. This synergistic effect of MDP with LPS or PGN was investigated in relation to expression and stability of TNF-α mRNA. Furthermore, the role of receptors (e.g. CD14 and TLR2/4) known to be involved in mediating cellular activation in response to bacterial cell wall components was studied.

EXPERIMENTAL PROCEDURES

Reagents—PGN from Staphylococcus aureus was obtained from BioCenika, MDP (N-acetylmuramyl-L-alanyl-D-isoglutamine) was from Calbiochem, Escherichia coli 055:B5 LPS and H-labeled E. coli K12 LCD25 LPS were from List Biological Laboratories, polymyxin B was from Bedford Laboratories, and actinomycin D was from Sigma. Affinity-purified anti-CD14 antibodies MEM-18 (IgG1) and MY4 (IgG2b) were purchased from SANBIO b.v. and Coulter, respectively. Functional grade purified anti-human TLR2 (clone TL2.1) and TLR4 (clone HTA125) antibodies (IgG2a) were from Biotechnology. Affinity-purified mouse IgG1 (Sigma), IgG2b (Coulter), and IgG2a (Sigma) were used as control antibodies. There were no effects of preincubation with these control antibodies for MEM-18, MY4, or TLRs. PGN was assayed for

1 The abbreviations used are: PGN, peptidoglycan; LPS, lipopolysaccharide; MDP, muramyl dipeptide (N-acetylmuramyl-L-alanyl-D-isoglutamine); TLR, Toll-like receptor; TNF, tumor necrosis factor; RT, reverse transcription; mAb, monoclonal antibody; IL, interleukin; sPGN, soluble PGN.

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Synergistic Effect of MDP with LPS and PGN

Endotoxin using the Limulus Amoebocyte Lysate assay (BioWhittaker). No significant endotoxin contamination of this preparation was detected (<1 ng of endotoxin/mg).

Cell Maintenance—Mono Mac 6 cells, provided by Dr. H. W. L. Ziegler-Heitbrock (University of Munich, Germany), were cultured in RPMI with 10% fetal calf serum (HyClone). Cells were cultured in a humidified incubator with 5% CO2 atmosphere at 37 °C. Cells were cultured in RPMI containing oxaloacetate, pyruvate, and bovine insulin, 10% fetal calf serum (HyClone). The cells were maintained in a humidified incubator with 5% CO2 atmosphere at 37 °C. New batches of frozen cell stock were grown up every 2 months, and growth morphology was evaluated. Before each experiment, Mono Mac 6 cells were allowed to differentiate for 2 days in the presence of 10 ng/ml calcitriol (Sigma). Cells were then incubated with each antibody for 30 min at 4 °C before incubation with E. coli LPS. MDP (final concentration 30 ng/ml) was first mixed with fetal calf serum (final concentration 7.5%) as a source for LPS-binding protein. It was added to the cells and incubated with frequent mixing for 1 h at 37 °C to reach equilibrium. Next, unlabeled LPS (final concentration 10 μg/ml) or a mixture of unlabeled LPS and MDP (final concentrations 10 and 100 μg/ml, respectively), were mixed first with fetal calf serum (final concentration 7.5%), were added to the cells. The cells were harvested at several time points. To determine nonspecific binding, unlabeled LPS alone or in combination with MDP was added to the cells before the addition of [3H]LPS. The cells were harvested by adding ice-cold HNE buffer (500 μl) and centrifuging the mixtures at 15,000 rpm for 2 min at 4 °C. The supernatant was aspirated, and the cells were lysed in 5 ml of liquid scintillation mixture (Beckman Instruments Inc.), and the cell-associated 3H was counted. Results represent means ± S.D. of triplicate samples. The experiment was repeated with similar results.

Data Analysis—LPS and PGN concentration-response data for stimulation of TNF-α production in Mono Mac 6 cells were analyzed using nonlinear least-squares curve fitting in Prism (GraphPad Software, Inc.). These data were fit with the logistic equation,

\[ Y = \frac{Y_m}{1 + \left(\frac{X}{EC_{50}}\right)^{Hill\ slope}} \]  

where \( Y \) represents the TNF-α response, \( X \) is the LPS or PGN concentration, \( Y_m \) is the maximum response, and \( EC_{50} \) is the concentration of LPS or PGN producing 50% stimulation.

[3H]LPS dissociation experiments were analyzed by fitting a monoequivalent decay equation to the data,

\[ Y = Y_m e^{-\frac{t}{k}} \]  

where \( Y \) is the amount of [3H]LPS bound at time \( t \), \( Y_m \) is the amount of [3H]LPS bound at time 0, \( k \) is the dissociation rate constant. Half-lives for [3H]LPS dissociation in the presence and absence of MDP were calculated as follows.

\[ t_{1/2} = 0.693/k \]  

RESULTS

Effect of MDP on the Responses of Mono Mac 6 Cells to LPS and PGN—The effect of a 30-min preincubation of Mono Mac 6 cells with MDP on TNF-α secretion induced by a wide concentration range of LPS and PGN was compared with incubation with either LPS or PGN alone (Fig. 1). Preincubation of cells with MDP resulted in a LPS concentration-response curve having a higher maximal level (a 2-fold increase), whereas the EC50 and Hill slope values did not differ significantly from those obtained in the absence of MDP. In the absence of MDP, the maximum concentration of produced TNF-α in response to LPS was 1477 pg/ml with a Hill slope of 2.7 and an LPS EC50 of 13.2 ng/ml. Pretreatment with MDP increased the maximum level of LPS-induced TNF-α production to 3003 pg/ml; the Hill slope and EC50 were 3.1 and 8.6 ng/ml, respectively. Stimulation with MDP alone resulted in a TNF-α concentration of 71 pg/ml. Similar results were obtained when cells were preincubated with MDP followed by PGN (Fig. 1b). The maximum level of TNF-α after incubation with PGN alone was 3313 pg/ml with a Hill slope of 1.1 and a PGN EC50 of 26.1 μg/ml. Preincubation with MDP yielded a maximum level of 5469 pg/ml, a Hill slope of 1.2, and an EC50 of 22.4 μg/ml. These results, derived from four-parameter logistic fits to the data, demonstrate that the singular observant effect of MDP is to increase the maximum TNF-α response of polyribosomal ribosomes to LPS or PGN in the absence of an influence on their respective potencies.

In addition to the experiments presented above, in which Mono Mac 6 cells were incubated with MDP before the addition of LPS or PGN, experiments also were performed in which MDP was added simultaneously with LPS or PGN. Simultaneous treatment of Mono Mac 6 cells with MDP and LPS or PGN
produced the same increase in TNF-α secretion (data not shown), suggesting that pretreatment was not necessary for the effect of MDP. Further, we performed several experiments in which the cells were first exposed to MDP for 10 or 20 min, washed, and then exposed to either LPS or PGN. The effect of exposure to MDP for only 10 min on TNF-α concentration was more than additive although not as dramatic as that occurring with simultaneous and continuous treatment of the cells with MDP and LPS or PGN (data not shown). We also performed experiments to determine whether preincubation with MDP had a synergistic effect with subsequent exposure to a second dose of MDP. In these experiments, the response was simply additive (data not shown).

To rule out the possibility that Mono Mac 6 cells incubated with MDP produce TNF-α that remains associated with the cells and was not secreted, TNF-α concentrations in the supernatant were compared with total (cell-associated and secreted) TNF-α concentrations. Incubation of cells with LPS, PGN, MDP, MDP plus LPS, and MDP plus PGN produced only small amounts of TNF-α that remained cell-associated; total TNF-α concentrations were indistinguishable from those in the supernatants (data not shown).

**Induction of TNF-α mRNA by LPS, PGN, and MDP—**TNF-α production in response to LPS is controlled both at the transcriptional and post-transcriptional levels (10–12). To study whether the synergistic induction of Mono Mac 6 cells by MDP is controlled at the level of gene transcription, TNF-α mRNA expression was measured after treatment with medium, LPS or PGN alone, or LPS or PGN in combination with MDP. The effects of LPS and PGN each were determined at their lowest concentrations causing maximal TNF-α protein production (i.e. LPS 30 ng/ml; PGN 100 μg/ml). Data presented in Fig. 2a indicate that incubation with LPS or PGN results in similar levels of TNF-α mRNA. Incubation with MDP (100 μg/ml) resulted in a 10-fold increase in TNF-α mRNA expression compared with control cells, although this was less than that caused by LPS or PGN. The same concentration of MDP caused very low levels of TNF-α protein secretion compared with LPS or PGN. Simultaneous treatment of cells with MDP and either LPS or PGN resulted in further enhancement of TNF-α mRNA expression, which appears to represent the additive effects of MDP and LPS or PGN individually. In contrast, concentrations of TNF-α protein in the supernatants from the same samples, even after only 90-min exposure, were suggestive of a pronounced synergistic effect of MDP with either LPS or PGN (Fig. 2b).

In addition to the experiment presented above, in which Mono Mac 6 cells were stimulated for 90 min before RNA extraction, the same experiments were also performed in which RNA was extracted after 30, 60, and 120 min of stimulation (Table I). In all cases, only a small amount of mRNA was expressed after 30 min. Stimulation with LPS or PGN produced maximal mRNA expression at 60 min, whereas stimulation with MDP or MDP in combination with LPS or PGN reached maximal values at 90 min. TNF-α mRNA expression...
was reduced markedly by 120 min. Varying the incubation period did not alter the finding that simultaneous treatment of cells with MDP and either LPS or PGN resulted in an additive effect on TNF-α mRNA expression.

**Comparison of MDP with LPS on TNF-α mRNA Expression and TNF-α Protein Concentration**—After determining that incubation of cells with MDP at 100 μg/ml resulted in TNF-α gene expression but minimal protein translation, we wondered whether this effect might be dependent on MDP concentration and whether the degree of mRNA expression in response to MDP might be insufficient to result in protein translation. To address this question, Mono Mac 6 cells were incubated for 90 min with medium alone as control, LPS ranging from 0.01 to 300 ng/ml, or MDP ranging from 0.01 to 300 ng/ml, or MDP ranging from 0.01 to 300 μg/ml. The half-lives of TNF-α mRNA in Mono Mac 6 cells stimulated with LPS, PGN, MDP, MDP plus LPS, and MDP plus PGN were 14.3, 14.6, 11.5, 14.1, and 13.4 min, respectively (Fig. 4). The half-life of TNF-α after stimulation with MDP alone was slightly shorter compared with the other stimuli, but the half-lives of the combinations of MDP and LPS or PGN did not differ significantly from those of LPS or PGN alone. Therefore, the effect of MDP with either LPS or PGN on release of TNF-α cannot be explained by altered stability of TNF-α mRNA. Consequently, other post-transcriptional factors must be responsible for the effect.

**Effect of Anti-CD14 mAbs on the Synergistic Effect of MDP**—The fact that maximal TNF-α mRNA expression for cells incubated with LPS or PGN occurred at 60 min whereas maximal mRNA expression for cells incubated with MDP alone or MDP with either LPS or PGN occurred at 90 min suggests that activation of gene expression by MDP might occur through a different route than LPS and PGN. To address this question, we performed experiments to explore the possibility that different receptors are involved in the response of the cells to MDP and either LPS or PGN. Two anti-CD14 mAbs, MY4 and MEM-18, were used to assess the involvement of CD14. It has been reported previously that the binding of LPS and PGN to CD14 involves amino acids 51–64 or 57–64 in the N-terminal region of the receptor (14, 15). MEM-18, with its epitope at residues 57–64, is an anti-CD14 mAb specific for this region. The epitope of MY4 is located closer to the N-terminal end of CD14 at amino acids 34–44. MY4 is quite effective in inhibit-

| TABLE I | Relative TNF-α gene expression by Mono Mac 6 cells after stimulation with different stimuli followed in time |  |
|---|---|---|---|---|---|---|---|
| | Relative quantity of TNF-α mRNA (95% confidence intervals) |  |
| | 30 min | 60 min | 90 min | 120 min |  |
| Untreated | 1.0–1.0 | 0.8–1.2 | 1.0–1.0 | 0.9–1.1 |  |
| LPS (30 ng/ml) | 1.1–1.6 | 17.3–18.1 | 15.8–19.4 | 11.5–12.5 |  |
| PGN (100 μg/ml) | 1.9–2.2 | 29.3–32.1 | 17.5–22.5 | 13.0–15.4 |  |
| MDP (100 μg/ml) | 0.9–1.5 | 3.2–4.2 | 7.2–8.5 | 2.4–2.6 |  |
| MDP (100 μg/ml) and LPS (30 ng/ml) | 1.2–1.4 | 21.2–22.5 | 26.8–33.6 | 8.9–11.7 |  |
| MDP (100 μg/ml) and PGN (100 μg/ml) | 2.0–2.3 | 22.4–30.4 | 27.1–31.2 | 14.2–18.6 |  |

**Fig. 3.** Comparison of MDP with LPS on TNF-α mRNA and TNF-α protein levels. Mono Mac 6 cells were stimulated with medium alone, increasing concentrations of LPS (0.01–100 ng/ml), or increasing concentrations of MDP (0.01–300 μg/ml) as indicated. After 90 min, TNF-α production was measured (a), and total RNA was extracted and subjected to RT-PCR analysis for detection of TNF-α mRNA (b).
ing binding of both LPS and sPGN to soluble CD14 (14). In experiments performed in our laboratory, both MEM-18 and MY4 completely neutralized the effect of LPS, whereas the effect of PGN was reduced by 86 and 54% by MEM-18 and MY4, respectively (Fig. 5). We have previously reported that increasing the concentration of these mAbs did not further increase their blocking effect on PGN (16). Even when LPS appeared to be completely blocked by the anti-CD14 mAbs, the presence of MDP resulted in significant amounts of TNF-α secretion (Fig. 5a). A similar effect of MDP on the response to PGN was observed, even when the effects of PGN alone were partially blocked with the anti-CD14 mAbs (Fig. 5b). These results suggest that the effect of MDP on the subsequent response to LPS and PGN was CD14-independent.

Expression of TNF-α mRNA was determined for cells incubated for 90 min with medium (control), LPS or PGN alone, or LPS or PGN in combination with MDP in the absence or presence of MY4. Whereas LPS-induced expression of TNF-α mRNA was almost completely abolished by MY4, this antibody had minimal effect on the responses to PGN or MDP alone, and the additive effects of MDP on the response to either LPS or PGN persisted in the presence of MY4 (Table II). These results provide further evidence that this effect of MDP was CD14-independent.

Influence of TLR4 and TLR2 on Synergism of MDP with LPS and PGN—TLR2 and -4 have been implicated as critical receptors responsible for initiation of signaling events and cellular activation in response to bacterial cell wall components (17). For instance, there is compelling information that TLR4 plays a critical role in LPS-induced cell signaling and serves as a cell surface co-receptor for CD14. These two receptors are necessary for LPS-mediated NF-κB activation and subsequent cellular events (18). Although incubation with the mAb directed against TLR4 reduced the effect of LPS by 80% (Fig. 6), it did not affect the synergism between MDP and LPS. MDP increased LPS-induced synthesis of TNF-α by 3.3-fold in the absence of the anti-TLR4 mAb and by 3.7-fold in the presence of the antibody. These findings provide evidence that the synergism of MDP with LPS was TLR4-independent.

The results of recent studies suggest that TLR2 is involved in cellular responses to a wide variety of infectious pathogens and their products, including PGN (17). Incubation of cells with the mAb against TLR2 reduced the effect of PGN by 51% (Fig. 7). In the absence of the anti-TLR2 mAb, MDP increased PGN-induced synthesis of TNF-α protein by 2.4-fold. Similarly, PGN-induced TNF-α production was increased 2.0-fold in cells co-incubated with MDP and the anti-TLR2 mAb. These findings suggest that the synergistic effect of MDP on the cellular response to PGN was also TLR2-independent.

Further, TNF-α mRNA expression was determined for cells incubated for 90 min with medium (control), LPS or PGN alone, or LPS or PGN in combination with MDP in the absence or presence of anti-TLR2 or anti-TLR4 mAb (Table III). Neither the anti-TLR2 nor the anti-TLR4 mAbs reduced TNF-α mRNA expression in response to MDP. Furthermore, the additive effects of MDP on expression of TNF-α mRNA induced by either LPS or PGN persisted in the presence of the anti-TLR mAbs.
providing additional evidence that MDP exerts its effect independent of TLR2 and TLR4.

**Effect of MDP on the [³H]LPS Off Rate from Mono Mac 6 Cells**—Based on the above results suggesting that MDP exerts its effects independent of CD14, TLR2, and TLR4, we considered the possibility that MDP might alter the interaction of LPS or PGN with these cell surface receptors. To investigate the possibility that MDP might exert an allosteric action to increase the affinity of LPS binding to the cell surface, the dissociation rate of LPS from the Mono Mac 6 cells was compared in the absence and presence of MDP (Fig. 8). The k of LPS was 0.0056 ± 0.0025 in the absence of MDP and 0.0061 ± 0.0023 in the presence of MDP. The t½ of LPS alone (125 min)

was not appreciably different from that of LPS in the presence of MDP (115 min).

**DISCUSSION**

In this study, we investigated the underlying mechanism for the apparent synergistic effect of MDP on TNF-α production induced by either LPS or PGN. Co-incubation of Mono Mac 6 cells with MDP and LPS yielded supernatant concentrations of TNF-α that were consistent with a synergism between the two bacterial components. We observed the same effect with co-incubation of the cells with MDP and PGN, a finding that has not been reported previously. Incubation of Mono Mac 6 cells with LPS or PGN alone showed a clear dose response for LPS and PGN starting at 2 ng/ml and 2 μg/ml, respectively. Concentrations of LPS (30 ng/ml) and PGN (100 μg/ml) resulted in peak supernatant concentrations of TNF-α that exceeded 1400 and 3300 pg/ml, respectively. In contrast, incubation of the cells with MDP alone at concentrations up to 300 μg/ml resulted only in slight increases in supernatant concentrations of TNF-α. The addition of MDP to either LPS or PGN resulted in substantial increases in TNF-α compared with LPS or PGN alone. This apparent synergistic effect was obvious with different concentrations of LPS and PGN, was evident after as little as 90 min of incubation, and was maintained throughout the 6-h incubation period. The presence of MDP significantly increased the maximum value of the dose-response curve for TNF-α secretion in response to LPS or PGN, without changing either the Hill slope or the EC₅₀. The primary effect of MDP was therefore to increase the maximum TNF-α response to LPS or PGN, which was not consistent with an allosteric action on either the LPS or PGN recognition site.

Others have reported a synergistic effect of muramyl peptides with LPS. Flak et al. (19) reported synergistic interactions between a naturally occurring PGN fragment (muramyl peptide) from Bordetella pertussis and LPS in the induction of inflammatory processes (induction of interleukin (IL)-1α, type II (inducible) nitric-oxide synthase, nitric oxide production, and inhibition of DNA synthesis) within hamster trachea epithelial cells. Yang et al. (4) reported the synergistic effect of MDP with LPS or lipoteichoic acid to induce inflammatory cytokine IL-8 in human monocytic cells in culture. Wang et al. (7) reported that co-administration of PGN or MDP with LPS caused significantly increased concentrations of TNF-α and IL-6 in cultures of whole human blood, whereas the release of IL-10 was not influenced. In contrast, we observed that TNF-α concentra-
tion in the supernatant of Mono Mac 6 cells co-incubated with the combination of LPS and PGN was only additive (data not shown). A possible explanation for this discrepancy is that we used insoluble PGN to eliminate potential effects of smaller PGN fragments on the responses being measured. Because Wang et al. (7) used a sonicated preparation of PGN, their PGN preparation may have included some smaller fragments that might act like MDP. Another difference between the two studies is the type of cell used; this may account for the differences noted. We report here for the first time that MDP, which is the minimum active fragment of PGN adjuvants (6), also synergizes with PGN in TNF-α production.

A unique finding of this study was that TNF-α mRNA expression showed a completely different profile than TNF-α protein production. Incubation of the Mono Mac 6 cells with MDP alone increased TNF-α mRNA, and co-incubation of the cells with MDP and either LPS or PGN resulted in TNF-α mRNA expression that approximated the sum of the message generated in response to MDP and either LPS or PGN alone. Although the additive effect on TNF-α mRNA expression was maximal at 90 min, the same trend was apparent regardless of the incubation period. With these data regarding the effects of MDP on TNF-α mRNA expression, the concentration of TNF-α protein simply reflects the additive effects of MDP and either LPS or PGN and not a synergistic interaction between these toxins. In short, co-incubation of MDP with either LPS or PGN increases TNF-α gene expression and TNF-α protein production to the same extent. Therefore, we conclude that TNF-α mRNA induced by MDP alone is not translated and that the impediment in translation is circumvented by the presence of either LPS or PGN.

Having identified the lack of correlation between expression of TNF-α mRNA and TNF-α protein induced by MDP and the subsequent responses to either LPS or PGN, we explored whether MDP mediates its effect through different cell surface receptors than LPS and PGN. It is well accepted that LPS and PGN initiate the production of proinflammatory mediators by interacting with the cluster differentiation antigen CD14 (20–23). CD14 is a glycosylphosphatidylinositol-anchored protein lacking transmembrane and cytoplasmic domains. There is compelling evidence that LPS and PGN transmit their signals via individual members of the TLR family, TLR4 (24, 25) and TLR2 (26, 27), respectively. An intermediary protein (MD-2) is required for interactions involving LPS, CD14, and TLR4 (28).

In our studies, we utilized two different anti-CD14 mAbs, MY4 and MEM-18, each of which completely blocked the response to LPS. In contrast, these antibodies only partially inhibited PGN-induced TNF-α production. However, neither antibody affected the apparent synergistic effect of MDP with either LPS or PGN, suggesting that LPS and PGN in the presence of antibodies that block CD14 were still able to initiate translation of the TNF-α mRNA induced by MDP. The latter finding indicates that the effect of MDP is CD14-independent. This CD14 independence was further confirmed by enhanced expression of TNF-α mRNA expression in response to MDP in the presence of MY4. The finding that PGN-induced synthesis of TNF-α was only partially blocked by MY4 and MEM-18 in this study was not completely unexpected. Dziarski et al. (14) have reported that MEM-18 almost completely inhibited binding of soluble CD14 to sPGN, when sPGN was immobilized on agarose. However, we determined in a previous study that PGN-induced TNF-α protein production in Mono Mac 6 cells could only be partially blocked by anti-CD14 mAbs, suggesting that PGN exerts its effect through CD14 and another unidentified receptor (16). This latter finding corroborates the inability of MY4 and MEM-18 to completely block the effects of PGN in the present study. The unidentified receptor could be TLR2, which may be activated by PGN independent of CD14 (29).

Unfortunately, commercially available anti-TLR mAbs do not completely block the effects of either LPS or PGN, probably because of partial neutralization by these antibodies. Nevertheless, the finding that these mAbs against TLR2 and TLR4 do not affect the apparent synergistic effect of MDP with either LPS or PGN strongly suggests that the effect of MDP is TLR2- and TLR4-independent. The strength of this conclusion was increased by the lack of effect of either the anti-TLR2 or the anti-TLR4 mAb on TNF-α mRNA expression by cells incubated with MDP.

Additional findings in the present study supporting our contention that MDP acts through receptor(s) other than those utilized by LPS and PGN are the following: 1) the fact that MDP increased the production of TNF-α protein induced by concentrations of LPS or PGN that by themselves already caused maximal TNF-α production, 2) the observation that maximal TNF-α mRNA expression occurs later when cells are incubated either with MDP alone or with MDP combined with either LPS or PGN than after stimulation with either LPS or PGN alone, and 3) the fact that the dissociation rate of LPS did not change in the presence of MDP, suggesting that MDP does not alter the interaction of LPS with its cell surface receptor, inasmuch as such an allosteric effect would have influenced [3H]LPS dissociation kinetics.

Our findings suggesting that MDP acts through receptors other than those utilized by LPS and PGN are in agreement with results reported for hamster tracheal epithelial cells and another monocytic cell line (4, 19). Although our finding that MDP acts independently of CD14 appears to contradict an earlier report that MDP binds to CD14 and prevents the binding of fluorescein isothiocyanate-labeled sPGN to human macrophages (30), the amount of MDP required to inhibit sPGN binding in that study was very high, indicating that MDP bound to those cells with low affinity. More recently, Dziarski et al. (14) reported that sCD14 can bind to MDP when coupled to agarose and that this binding could not be inhibited by monomeric soluble MDP. Those investigators concluded that polymeric, aggregated or solid-bound PGN or MDP is needed for CD14 binding, which is in agreement with our findings that the effect of MDP is CD14-independent.

What remains to be determined is the mechanism(s) responsible for inhibiting translation of the TNF-α mRNA expressed in response to MDP alone and the pathway of cellular activation induced by MDP. It is well known that the synthesis of TNF-α is tightly controlled at many different levels and is subject to several negative feedback mechanisms (11, 31). For example, post-transcriptional regulation of TNF-α production is mediated by the AU-rich element located in the TNF-α mRNA 3'-untranslated region, which controls its translation and stability (32). Metabolism of the 3' poly(A) tail region of TNF-α mRNA plays a critical regulatory function in TNF-α translation (33). In unstimulated but adherent cells, shortening of the length of the poly(A) tail prevents the initiation of TNF-α translation. This process is reversed by LPS, allowing synthesis of translatable polyadenylated TNF-α mRNA. It is possible that MDP lacks the ability to exert the same effect as LPS, rendering mRNA transcribed in response to MDP in a form that requires an additional stimulus (e.g. LPS) in order for protein translation to occur. The end result of cellular activation by LPS or PGN is up-regulation of more than 120 genes, including differential activation of MAP kinases and slightly different patterns of gene activation (34). It is possible that
activation of some of these genes is important for translational control of TNF-α production and that these genes are not activated by MDP alone.

In an effort to explain the apparent synergistic effect of MDP and LPS on TNF-α protein production, some possibilities have been addressed by other investigators. Wang et al. (7) reported a 2-fold increase in surface expression of CD14 on monocytes and suggested that both PGN and MDP prime leukocytes for LPS-induced release of proinflammatory cytokines. However, this effect could not explain the observed synergism between MDP and LPS or MDP and PGN, since up-regulation of CD14 occurred after the synergistic effect was evident. Other investigators have suggested involvement of MyD88, an adapter molecule essential for cell signaling events initiated via the TLR family (35). In this study, we have shown that the inability to translate mRNA induced by MDP is not due to alterations in mRNA stability. We also determined that there were no differences in late mRNA induced by MDP is not due to alterations in mRNA expression in THP-1 cells, but there was no synergistic up-regulation of MyD88 mRNA in cells incubated with the combination of MDP and LPS (4). Furthermore, the up-regulation of MyD88 that was detected occurred later than the synergistic effect and so could not account for the synergism. In this study, we have shown that the inability to translate mRNA induced by MDP is not due to alterations in mRNA stability. We also determined that there were no differences in transport between TNF-α induced by MDP and LPS or PGN, since secreted TNF-α concentrations were indistinguishable from total TNF-α concentrations.

In summary, we have demonstrated for the first time that MDP induces TNF-α gene expression without significant TNF-α translation. Furthermore, the block in translation is removed in the presence of LPS and PGN, thereby accounting for the apparent synergistic effect of MDP on TNF-α protein production. The amount of TNF-α protein produced in response to the combination of either MDP and LPS or MDP and PGN is expected in light of the amount of TNF-α mRNA expressed individually in response to MDP, LPS, or PGN. Furthermore, we have demonstrated that the effect of MDP alone and its apparent synergism with either LPS or PGN is CD14-, TLR2-, and TLR4-independent. Thus, we conclude that MDP exerts its effects via different receptors than those responsible for the effects of LPS and PGN. Taken in the context of reports documenting the deleterious effects of MDP in animals subjected to experimentally induced septicemia (36) and the fact that antimicrobial drugs exert their effects by causing the breakdown of bacterial cell wall components such as PGN (37), the results of the current study underscore the importance of considering both serum concentrations of inflammatory mediators and gene expression profiles when interpreting findings associated with septicemia.

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