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G(Anh)MTetra, a Naturally Occurring 1,6-Anhydro Muramyl Dipeptide, Induces Granulocyte Colony-Stimulating Factor Expression in Human Monocytes: a Molecular Analysis

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***N*-Acetylglucosaminyl-1,6-anhydro-*N*-acetylmuramyl-L-alanyl-D-isoglutamyl-*m*-diaminopimelyl-D-alanine [G(Anh)MTetra], a naturally occurring breakdown product of peptidoglycan from bacterial cell walls, was studied for its ability to induce granulocyte colony-stimulating factor (G-CSF) mRNA and protein expression in human adherent monocytes. Resting monocytes did not express G-CSF mRNA or secrete G-CSF protein. In contrast, monocytes exposed to G(Anh)MTetra showed a dose-dependent increase in G-CSF mRNA accumulation, which correlates with the secretion of G-CSF protein. Maximal levels of G-CSF mRNA were reached within 2 h of activation. Expression of G-CSF was mediated by an increase in the stability of G-CSF transcripts rather than by an increase in the transcription rate of the G-CSF gene. Experiments with the protein synthesis inhibitor cycloheximide revealed that G(Anh)MTetra-induced G-CSF mRNA expression was independent of new protein synthesis. Furthermore, it was shown that the effect of G(Anh)MTetra was regulated by a protein kinase C-dependent pathway, whereas protein kinase A and tyrosine kinases were not involved. Finally, it was shown that G(Anh)MTetra also induced G-CSF mRNA expression in human endothelial cells. The data indicate that, besides lipopolysaccharide, other naturally occurring bacterial cell wall components are able to induce G-CSF expression in different hematopoietic cells.**

Granulocyte colony-stimulating factor (G-CSF) is an important hematopoietic growth factor that alters the function and augments the production of polymorphonuclear cells in vivo and in vitro (6). During gram-negative bacterial infection, G-CSF protein levels are elevated (19) because of stimulatory effects of lipopolysaccharide (LPS) on monocytes and endothelial cells resulting in increased expression of G-CSF at the mRNA and protein levels (29). However, granulocytosis is also observed during infections with gram-positive bacteria, indicating that additional factors are of importance for controlling G-CSF expression.

N-Acetylglucosaminyl-1,6-anhydro-*N*-acetylmuramyl-L-alanyl-D-isoglutamyl-*m*-diaminopimelyl-D-alanine [G(Anh)MTetra] is a naturally occurring bacterial cell wall breakdown product that is produced by the soluble lytic transglycosylases of *Escherichia coli* (13, 17). It is structurally similar to the muramylpeptides produced by lysozyme but possesses an internal 1,6-anhydro bond within the muramic acid residue (14, 17). G(Anh)MTetra has been shown to be released during normal growth by *Neisseria gonorrhoeae* (24, 27) and has been shown to be identical to tracheal cytotoxin of *Bordetella pertussis* (5) and to the urinary sleep-inducing factor (20, 21, 23). The data suggest that G(Anh)MTetra can be an additional factor that modulates cytokine expression during an inflammatory response.

To obtain quantities large enough to perform the experiments, we made use of a recently described method to isolate G(Anh)MTetra by using immobilized murein hydrolases from *E. coli* fused to staphylococcal protein A (14). In the present study, we show that G(Anh)MTetra strongly induces G-CSF mRNA and protein expression in human monocytes, which is regulated at a posttranscriptional level. G(Anh)MTetra was also found to induce G-CSF mRNA expression in endothelial cells. The results suggest that the release of G(Anh)MTetra by bacteria is a cofactor in promoting granulocytosis during bacterial infections.

MATERIALS AND METHODS

Preparation of cells. Peripheral blood cells were obtained from volunteer platelet donors, and mononuclear cell suspensions were prepared by Ficoll-Hypaque density gradient centrifugation. T lymphocytes were removed by 2-aminoethylisothiouronium bromide-treated sheep erythrocyte rosetting. Monocytes were further enriched by plastic adherence (1 h, 37°C) to a purity of >95%, as detected by fluorescence-activated cell sorter analysis with anti-CD-14 (Beckton-Dickinson, Sunnyville, Calif.) antibody. Monocytes were cultured at a density of 1×10^6 to 2×10^6/ml in RPMI 1640 medium (Flow, Rockville, Md.) with 10% fetal bovine serum, supplemented with 100 U of penicillin per ml, 100 μg of streptomycin per ml, 2 mM L-glutamine, and 6 ng of colistin per ml.

Human umbilical vein endothelial cells were harvested according to the method of Jaffe et al. (18), except that 0.05 mg of chymotrypsin per ml (Sigma, St. Louis, Mo.) resuspended in 0.1 mol of phosphate-buffered saline per liter was used instead of collagenase. This provides a higher yield of viable endothelial cells with fewer contaminating smooth muscle cells. Primary isolates were cultured in gelatin (Sigma)-precoated 25-cm^2 tissue culture flasks (Costar, Cambridge, Mass.) at 37°C. Culture medium was RPMI 1640 supplemented with 20% heat-inactivated human pooled serum, 2 mM L-glutamine, 5 U of heparin (Leo Pharmaceutical Products B.V., Weesp, The Netherlands) per ml, 50 μg of endothelial cell growth factor

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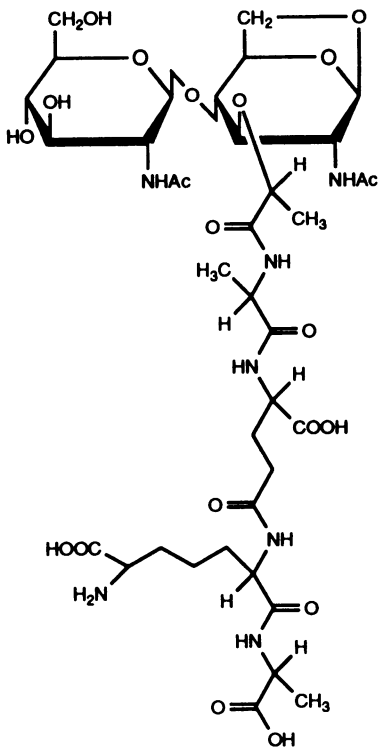

FIG. 1. Structure of monomeric 1,6-anhydro-muropeptide G(Anh) MTetra.

supplement (extracted from bovine hypothalamus) per ml (22), 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Two confluent primary cultures were trypsinized, pooled, and subcultured into 75-cm^2 tissue culture flasks. The cells were characterized morphologically by growth contact inhibition and by their typical cobblestone appearance at confluence and were characterized immunologically by indirect immunofluorescence of intracellular von Willebrand factor and positive staining with the endothelial cell-specific surface marker CLB-HEC-19 (28).

Isolation of G(Anh)MTetra. G(Anh)MTetra was enzymatically prepared by using immobilized murein hydrolases from *E. coli* fused to staphylococcal protein A as described previously (14). Five micrograms of G(Anh)MTetra contained less than 50 pg of endotoxin as measured by a chromogenic *Limulus* amoebocyte lysate assay as described previously (9). The purity of the preparation was determined with high-performance liquid chromatography analysis as described and was 90%. The other 10% consisted of 1,6-anhydro-muramyl peptides with a different peptide moiety (14). The concentration was determined by amino acid analysis after acid hydrolyzation and *ortho*-phthaldialdehyde on a Hewlett-Packard analyzer. The structure of G(Anh)MTetra is depicted in Fig. 1.

Stimulation. Before activation, monocytes were kept in the described medium for 16 h. Subsequently, monocytes were treated for various time periods with 1.25 μg of G(Anh) MTetra per ml. H7 (Calbiochem, La Jolla, Calif.) was used at 50 μM, H8 (Calbiochem) was used at 1.4 μM, and genistein (Sigma) was used at 20 μg/ml. Cycloheximide (CHX [Sigma]) and actinomycin D (Act D [Boehringer, Mannheim, Germany]) were used at 10 μg/ml. Endothelial cells were stimulated as confluent monolayers of the second passage for 6, 12, and 24 h with 1.25 μg of G(Anh)MTetra per ml in RPMI 1640 medium supplemented with 10% heat-inactivated pooled human serum.

mRNA extraction and analysis. Total cellular RNA was isolated and G-CSF mRNA levels were analyzed by Northern (RNA) analysis as described previously (12) by using a 1,600-nucleotide ^{32}P-labeled G-CSF cDNA probe (gift from S. C. Clark, Genetics Institute, Cambridge, Mass.). To demonstrate that equal amounts of RNA were loaded, the filter was stripped in 0.1% sodium dodecyl sulfate and rehybridized with a 7.8-kb ^{32}P-labeled 28S DNA probe. Hybridization, washing, and exposure of the membranes were performed as described previously (12). Quantification of mRNA levels was performed by densitometry with a Gel Scan laser densitometer (Pharmacia LKB, Uppsala, Sweden).

G-CSF secretion. A total of 10^6 monocytes of three different individuals were cultured in RPMI 1640 medium with 10% fetal bovine serum during 48 h or in medium in the presence of G(Anh)MTetra (1.25 μg/ml) during 48 h. After the culture period, cell supernatants were analyzed for G-CSF protein levels with an enzyme-linked immunosorbent assay (ELISA [ITK Diagnostics, Minneapolis, Minn.]) as recommended by the manufacturer. The detection limit of the assay is 25 pg/ml. The Student *t* test for paired samples was used to determine the significance of the secretion data.

Nuclear run-on assay. The run-on analysis was performed as previously described (11) with pooled nuclei from three donors. Monocytes were treated with medium (control) or 1.25 μg of G(Anh)MTetra per ml for 1 or 2 h. Five micrograms of each of the following DNAs was immobilized on Hybond N^+ membranes: (i) *Eco*RI-linearized pGEM (negative control); (ii) *Eco*RI-linearized plasmid containing a 1.6-kb fragment of the human G-CSF cDNA; (iii) *Eco*RI-linearized plasmid containing a 1.2-kb fragment of the human c-*jun* cDNA (gift from R. Henschler, Freiburg, Germany) (1); (iv) *Eco*RI-linearized plasmid containing a 1.3-kb rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA fragment (16). Hybridization of labeled RNAs to these membranes was performed as described previously (11). Quantification of transcription rate levels was performed by densitometry with a Gel Scan laser densitometer (Pharmacia LKB).

RESULTS

G(Anh)MTetra induces G-CSF protein secretion by monocytes. Monocytes of three different donors were treated with or without G(Anh)MTetra during 48 h. Cell supernatants were collected and assayed for G-CSF protein by ELISA. In agreement with previous reports, resting monocytes secreted no or very little G-CSF protein (45.3 ± 7.8 pg/ml [mean ± standard deviation; $n = 3$]). In contrast, G(Anh)MTetra-treated monocytes secreted significant ($P < 0.025$) levels of G-CSF (884.3 ± 322.4 pg/ml [mean ± standard deviation; $n = 3$]). To study the molecular mechanisms involved in the induction of G-CSF secretion by G(Anh)MTetra, the next set of experiments were performed.

Kinetics of G(Anh)MTetra-induced G-CSF mRNA expression. Monocytes were treated with G(Anh)MTetra during 0, 2, 4, and 8 h. Total cellular RNA was isolated and subjected to Northern analysis. Untreated monocytes did not express detectable levels of G-CSF mRNA (Fig. 2). However, upon activation with G(Anh)MTetra, a strong increase in G-CSF mRNA accumulation was noticed. Maximal levels were observed after 2 h of activation (Fig. 2A). G-CSF mRNA expression returned to undetectable levels after 4 and 8 h of G(Anh)MTetra treatment (data not shown). To demonstrate that G-CSF mRNA expression was dependent on the dose of

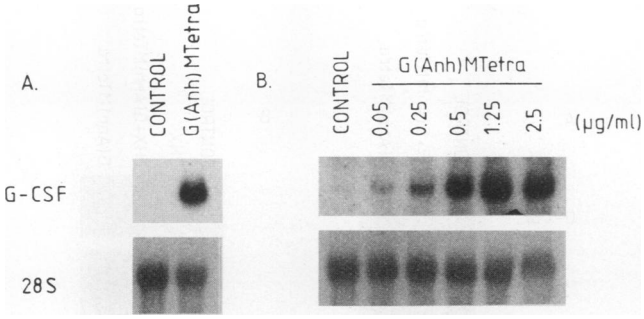

FIG. 2. Effect of G(Anh)MTetra on G-CSF mRNA expression in human monocytes. (A) Northern analysis of total monocyte RNA extracted after incubation in medium (control) or after incubation with 0.5 μg of G(Anh)MTetra per ml for 2 h. The 28S signal demonstrates equal gel loading. The results shown are from one of two experiments performed. (B) Dose-response curve of G(Anh)MTetra's effect on G-CSF mRNA accumulation. Northern analysis results with total monocyte RNA extracted from monocytes treated for 2 h with increasing concentrations of G(Anh)MTetra are shown. The 28S signal demonstrates equal gel loading. The results shown are from one of two experiments performed.

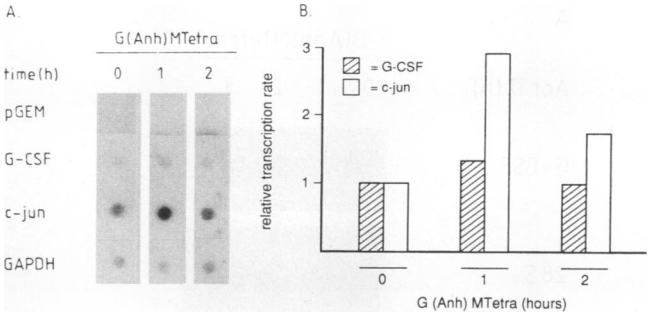

FIG. 3. Effect of G(Anh)MTetra on the transcription rate of the G-CSF gene in human monocytes. (A) Nuclear run-on experiment. Nuclei were prepared from monocytes that were treated with medium (control) and with 1.25 μg of G(Anh)MTetra per ml for 1 and 2 h. Transcription in the isolated nuclei was analyzed by hybridization of the ^{32}P transcripts to 5 μg of linearized plasmids containing no insert (pGEM), G-CSF, c-*jun*, and GAPDH cDNA inserts immobilized on nylon membranes. The experiment shown is representative of two experiments performed. (B) Schematic representation of G-CSF and c-*jun* transcription rates in control and G(Anh)MTetra-treated monocytes. Quantification of transcription rates was performed by densitometry, and rates were normalized with respect to the GAPDH signal.

G(Anh)MTetra used, Northern analysis was performed with RNA isolated from monocytes stimulated with increasing amounts of G(Anh)MTetra. As shown in Fig. 2B, G-CSF mRNA expression was dependent on the dosage used and was optimal at 1.25 μg/ml. Subsequently, in the next set of experiments, monocytes were activated with 1.25 μg of G(Anh)MTetra per ml.

G(Anh)MTetra-induced G-CSF mRNA expression is not mediated by an increase in G-CSF gene transcription. Nuclear run-on assays were performed to define whether the stimulatory effects of G(Anh)MTetra could be ascribed to an increased transcription rate. Because of the relatively fast response to G(Anh)MTetra, resulting in maximal G-CSF mRNA accumulation at 2 h, transcription rates were analyzed after 1 and 2 h of treatment. Two independent experiments demonstrated that the transcription rate of the G-CSF gene was not significantly affected by G(Anh)MTetra (Fig. 3), whereas the c-*jun* transcription rate was enhanced by G(Anh)MTetra by a factor of 3 after 1 h of treatment. Therefore, the cytoplasmic stability of G-CSF transcripts in G(Anh)MTetra-treated monocytes was studied.

G(Anh)MTetra stabilizes G-CSF mRNA. Monocytes were activated with G(Anh)MTetra for 2 h. Act D was then added to stop new RNA synthesis. At several time points after Act D addition, total cellular RNA was prepared and subjected to Northern analysis. Previously, it was shown that G-CSF mRNA in resting monocytes is short-lived, with a message half-life of less than 15 min (15). In contrast, as demonstrated in Fig. 4, G(Anh)MTetra-induced G-CSF mRNA decayed with a half-life of approximately 2.5 h, indicating that posttranscriptional mechanisms are involved in G(Anh)MTetra-induced G-CSF expression.

Expression of G(Anh)MTetra-induced G-CSF mRNA is through activation of a PKC-dependent pathway and does not depend on new protein synthesis. To study the involvement of secondary pathways in the induction of G-CSF mRNA by G(Anh)MTetra, we treated monocytes with H7, H8, and genistein to block protein kinase C (PKC), protein kinase A, and tyrosine-dependent kinases, respectively. Total RNA was isolated and subjected to Northern analysis. Figure 5 shows that a preincubation with H7 totally abolished the G(Anh)MTetra-induced G-CSF mRNA expression. In contrast, H8 and genistein had no or little effect. These results indicate that G(Anh)MTetra-induced G-CSF mRNA expression is regulated through activation of a PKC-dependent pathway. As shown in Fig. 5, treatment with the protein synthesis inhibitor CHX superinduced G-CSF mRNA expression. Furthermore, G(Anh)MTetra plus CHX-induced G-CSF mRNA levels were enhanced compared with the effect of G(Anh)MTetra alone, indicating that G(Anh)MTetra-induced G-CSF mRNA expression does not depend on the synthesis of new protein.

G(Anh)MTetra induces G-CSF mRNA in endothelial cells. Human umbilical vein endothelial cells are known to express G-CSF after activation in vitro (4, 29), and, therefore, it is likely that endothelial cells contribute to the total amount of G-CSF that circulates. To study the effect of G(Anh)MTetra on G-CSF expression in endothelial cells, we performed Northern analysis of total RNA isolated from endothelial cells treated for 6, 12, and 24 h with G(Anh)MTetra. As shown in Fig. 6, G-CSF mRNA is expressed after 12 and 24 h of stimulation with G(Anh)MTetra.

DISCUSSION

In this paper, we demonstrated that G(Anh)MTetra, a naturally occurring bacterial cell wall breakdown product, induces G-CSF expression in human monocytes and endothelial cells. A distinct difference between the kinetics of G-CSF mRNA expression in both cell types was observed. In monocytes, G-CSF mRNA appeared within 2 h of stimulation, whereas in endothelial cells G-CSF mRNA appeared after 12 h. In monocytes, G-CSF mRNA expression was induced by a PKC-dependent signaling pathway and was independent of the synthesis of new protein. In agreement with our results, it has been shown that G-CSF expression is mainly regulated by posttranscriptional mechanisms (7, 15). De Wit et al. demonstrated a stabilizing effect of gamma interferon on LPS-induced G-CSF transcripts in human monocytes (7). Earlier, Ernst et al. showed that LPS-induced G-CSF mRNA expression is not mediated through an enhanced transcription rate of the G-CSF gene but rather is due to stabilization of G-CSF mRNA (15). In view of these results, our findings suggest that

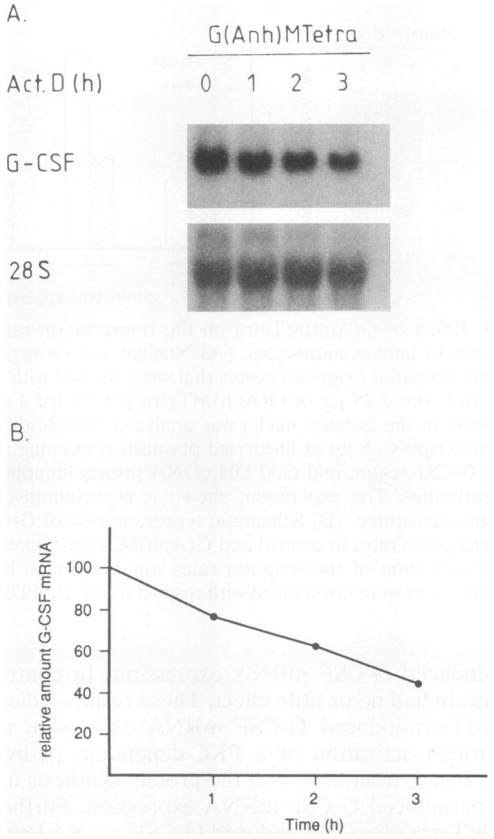

FIG. 4. Effect of G(Anh)MTetra on the stability of G-CSF mRNA in human monocytes. Monocytes were cultured for 2 h with G(Anh) MTetra. Act D (10 μg/ml) was added to inhibit transcription. (A) Northern analysis was performed to determine G-CSF mRNA and 28S rRNA levels at various time points (time indicated in hours) after Act D addition. mRNA half-life was determined after quantification of G-CSF mRNA levels by densitometry after normalization with respect to the 28S signal. (B) Percentage change of G-CSF mRNA accumulation over time.

G(Anh)MTetra induces a preexisting protein (presumably by modifying the protein) that protects G-CSF mRNA from degradation.

With regard to the intracellular pathway that regulates G-CSF mRNA expression, we and others previously showed that monocytes express G-CSF mRNA upon activation with the calcium ionophore A23187, diacylglycerol, or LPS (7, 15, 29, 30), all of which activate PKC-dependent pathways (3, 32). Furthermore, inactivation of PKC by pretreatment with phorbol myristate acetate strongly inhibited LPS-induced G-CSF secretion in human monocytes (7). These results correlate with our present finding that H7, which blocks PKC activity, abolished G(Anh)MTetra-induced G-CSF mRNA expression. G-CSF mRNA is also induced by blocking protein synthesis (15 [and our results]). A short-lived protein that degrades the G-CSF transcript is thought to disappear upon CHX treatment (15). This short-lived protein does not play a role in G(Anh)MTetra-induced G-CSF mRNA expression, since CHX also enhanced the G(Anh) MTetra-induced G-CSF mRNA expression.

A synthetic muramyl dipeptide, *N*-acetyl-muramyl-L-Ala-D-iso-Gln (MDP) is a powerful tool to stimulate host defense (2). Treatment of monocytes and monocytic cell lines with MDP

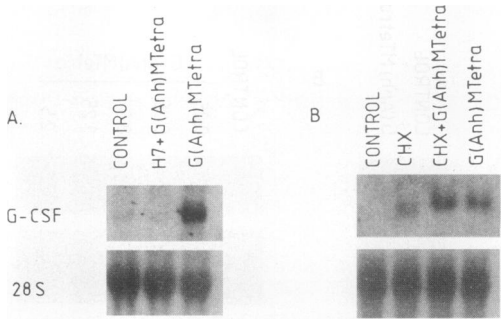

FIG. 5. Effect of specific inhibitors on G(Anh)MTetra-induced G-CSF mRNA expression in human monocytes. Results of Northern analysis of total RNA extracted from monocytes treated for 30 min with the indicated inhibitors and subsequently with 1.25 μg of G(Anh) MTetra per ml for 2 h are shown. The 28S signal demonstrates equal gel loading. The results shown are from one of two experiments performed.

and lipophilic MDP derivatives has also been used as a model to study the induction of cytokine expression upon challenge with bacterial cell wall products (2). For instance, MDP-Lys (L-18) was shown to induce G-CSF mRNA and protein expression in human peripheral blood mononuclear cells (26). However, MDP is not a naturally occurring muramyl peptide. MDP is not produced after phagocytosis by macrophages, and it is not a product of peptidoglycan-degrading enzymes (25, 31). Therefore, physiological studies of the relevance of cytokine production and other factors induced by bacterial products preferably should be performed with natural bacterial breakdown products rather than synthetic compounds.

Exposure of human monocytes to LPS rapidly induces expression of G-CSF (7, 15, 29, 30) with no effect on the expression of macrophage CSF. Similarly, G(Anh)MTetra induces G-CSF, whereas monocyte CSF mRNA expression was not induced (unpublished results). Taken together, the data indicate that a bacterial infection, involving the release of either LPS or G(Anh)MTetra, might promote granulocytosis. So far, release of G(Anh)MTetra is only described for gram-negative bacteria. However, it seems likely that G(Anh) MTetra molecules or closely related molecules are breakdown products of gram-negative and gram-positive bacteria. From the results of this study, it can be hypothesized that G(Anh) MTetra or closely related bacterial components play a role in the activation of monocytes and endothelial cells in vivo, thereby inducing the expression of several cytokines. Indeed,

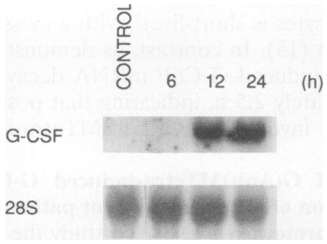

FIG. 6. Effect of G(Anh)MTetra on G-CSF mRNA expression in human endothelial cells. Results of Northern analysis of total RNA extracted from endothelial cells treated for 6, 12, and 24 h with 1.25 μg of G(Anh)MTetra per ml are shown. The 28S signal demonstrates equal gel loading. The results shown are from one of two experiments performed.

G(Anh)MTetra was identified as a strong inducer of interleukin 1 expression in human peripheral mononuclear cells (8) and interleukin 1 and interleukin 6 expression in human monocytes (10). Therefore, in conjunction with LPS, G(Anh)MTetra might be a cofactor in stimulation of cytokine production. In view of these data, it is conceivable that monoclonal antibodies directed against LPS can only partially modulate the clinical course of patients with gram-negative bacteremia. Therefore, it would be interesting to study monoclonal antibodies directed against 1,6-anhydro-muramyl peptides in conjunction with anti-LPS monoclonal antibodies for their effect on mortality in patients suffering from bacterial infection.

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