Chemokines Are the Main Prolinflammatory Mediators in Human Monocytes Activated by *Staphylococcus aureus*, Peptidoglycan, and Endotoxin*

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It is widely believed that the cytokines tumor necrosis factor (TNF-α), interleukin (IL)-1, and IL-6 are the main proinflammatory mediators induced in the host by bacteria and their cell wall components. To test this hypothesis, we compared the level of expression of 600 genes activated in human monocytes by *Staphylococcus aureus*, peptidoglycan, endotoxin, and interferon-γ. These stimulants induced expression of over 120 genes, as identified by cDNA arrays. The highest activated genes for proinflammatory mediators induced by all three bacterial stimulants were chemokine genes (IL-8 and macrophage inflammatory protein (MIP)-1α), whereas cytokine genes (TNF-α, IL-1, and IL-6) were induced to a lower extent. Genes for other chemokines (MIP-2α, MIP-1β, and monocyte chemoattractant protein-1) were also induced higher than the cytokine genes by peptidoglycan, and as high or higher than the cytokine genes by *S. aureus* and endotoxin. This high induction of chemokine genes was confirmed by quantitative RNase protection assay, and high secretion of chemokines was confirmed by enzyme-linked immunosorbent assays. Although genes for chemokines were the highest and genes for cytokines were the second highest induced genes by all three bacterial stimulants, each stimulus induced a unique pattern of gene expression. By contrast, expression of a completely different gene pattern was induced by a nonbacterial stimulus, interferon-γ. These results establish chemokines as the main mediators induced by both Gram-positive and Gram-negative bacteria and are consistent with the highly inflammatory nature of bacterial infections.

Infections with both Gram-positive and Gram-negative bacteria have similar main clinical manifestations, such as inflammation, fever, leukocytosis, hypotension, decreased peripheral perfusion, malaise, decreased appetite, sleepiness, and arthritis, which are caused by mediators released from host cells exposed to bacterial cells and their components (1, 2). It is widely accepted that the main proinflammatory mediators induced by bacteria and their cell wall components are cytokines, primarily TNF-α, IL-1, and IL-6 (3–7). The main bacterial components responsible for the induction of these clinical manifestations are endotoxin (lipopolysaccharide (LPS)) in Gram-negative bacteria and peptidoglycan (PGN) and lipoteichoic acid in Gram-positive bacteria (1–5, 8). The main target cells activated by these bacterial components are monocytes and macrophages, which are activated through two pattern recognition receptors, CD14 and Toll-like receptors (TLRs) (1, 2, 9–17).

So far, only bacterially induced expression of individual genes or of small groups of genes has been studied, and expression of a large number of genes induced by bacterial and nonbacterial activators has not been simultaneously compared. Therefore, the objective of this study was to determine the expression patterns of a large number of genes in human monocytes exposed to bacteria or to the main components of Gram-positive and Gram-negative bacteria (PGN and LPS, respectively), using a 600-cDNA gene array. This unbiased approach was then used to compare the gene expression patterns induced by bacterial and nonbacterial monocyte activators and to measure which genes are the most strongly induced by bacterial and nonbacterial activators. Since the highest induced genes were the genes for proinflammatory mediators, we then compared the amounts of different mediators induced by bacterial stimulants.

**EXPERIMENTAL PROCEDURES**

**Stimulants**—Soluble PGN was purified by vancomycin affinity chromatography from *Staphylococcus aureus* Rb and contained <24 pg of endotoxin/mg of PGN (17). *S. aureus* Rb cells were killed with gentamicin (800 μg/ml, 37 °C, 2 h) and contained <10 pg of endotoxin/10⁶ cells (18). LPS from *Salmonella minnesota* Re 595 (a minimal naturally occurring endotoxic structure of LPS) was from Sigma. Human recombinant interferon-γ (IFN-γ) was produced in baculovirus-infected *Trichoplusia ni* cells (specific activity, 0.8–4 × 10⁶ units/ml; endotoxin content, <0.15 ng/μg; Pharmingen, San Diego, CA).

**Cell Cultures**—Human blood monocytes, obtained from healthy males (24–49 years old of Caucasian, Asian, or African-American origin) by Histopaque (density 1.077 g/ml; Sigma) centrifugation and adherence to plastic (19) (>95% pure by phagocytosis of Latex and non-specific esterase), were cultured at 10⁵ cells/cm² in Falcon 3003 plates (for RNA isolation) or 96-well plates (for enzyme-linked immunosorbent assays) in RPMI 1640 with 10% autologous or pooled human donor AB serum (Sigma). Stable CD14 transfectants of human monocytic cell line

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1 The abbreviations used are: TNF, tumor necrosis factor; IFN, interferon; IL, interleukin; IP, interferon-γ-inducible protein; LPS, lipopolysaccharide; MCP, monocyte chemoattractant protein; MIG, monokine induced by IFN-γ; MIP, macrophage inflammatory protein; MRP, macrophage inhibitory factor-related calcium-binding protein (calcigranulin); PGN, peptidoglycan; RANTES, regulated on activation, normal T expressed and secreted; TLR, Toll-like receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Induction of Chemokines by Bacterial Components

THP-1/CD14 (20), obtained from Peter Tobias (Scripps Research Institute, La Jolla, CA), were cultured in RPMI 1640 with 10% fetal calf serum. Medium (control) or optimal stimulatory concentrations of PGN (10 μg/ml), *S. aureus* (4 × 10^8 cells/ml), LPS (10 μg/ml for monocytes and 100 μg/ml for THP-1/CD14 cells), or IFN-γ (100 μg/ml) were added for the lengths of time indicated under “Results.”

**RNA Preparation and cDNA Synthesis**—Total RNA was extracted by phenol-chloroform method (21) and digested for 1 h at 37 °C with DNase I using the MessageClean kit (Gene Hunter, Nashville, TN). Poly(A)^+ RNA was extracted by oligo(dT)-cellulose chromatography using the Qiagen (Valencia, CA) OligoTect mRNA Mini Kit. To synthesize cDNA from poly(A)^+ RNA, 1 μg of poly(A)^+ RNA was mixed with 1 μl of 10× CDS primer mix, incubated in a preheated thermal cycler at 70 °C for 2 min and at 50 °C for 2 min and then incubated at 50 °C for 25 min with a mixture of 5× reaction buffer, 10× dNTP, 300 Ci/ml [α-32P]dATP, 0.5 μl of 100 molar dithiothreitol, and 50 units of Moloney murine leukemia virus reverse transcriptase (Clontech, Palo Alto, CA) in a total volume of 10 μl. The reaction was stopped by adding 1 μl of 10× termination mix, and cDNA was purified on a Chroma Spin-200 column (Clontech).

**cDNA Array Hybridization**—The Atlas human cDNA array membranes (Clontech) contained 588 inducible test genes (including oncogenes, tumor suppressors, cell cycle regulators, ion channels and transport, intracellular signal transduction modulators and effectors, stress response, apoptosis, DNA synthesis, repair, and recombination, transcription factors and other DNA-binding proteins, receptors, cell surface antigens, cell adhesions, and cell-cell communication proteins), nine housekeeping genes, and three negative controls (the list of genes, their position on the membrane, and their GenBank™ accession numbers can be obtained on the World Wide Web). Prehybridization was performed at 68 °C for 30 min in 10 ml of ExpressHyb buffer with 1.5 mg of sheared salmon sperm DNA for 30 min and hybridization was performed at 68 °C overnight with 1–5 × 10^6 cpm of denatured cDNA probe, as recommended by the manufacturer. The membranes were washed at 68 °C to a final stringency of 0.1× SSC, 0.5% SDS, and subjected to autoradiography. Each spot was quantified by Kodak Digital Science Image Station 440CF and Image Analysis Software version 3.0. The results were then normalized based on hybridization to two housekeeping genes (actin and ribosomal protein S9) whose expression did not change upon stimulation (with the exception of IFN-γ, for which only S9 was used, because expression of actin mRNA was enhanced by IFN-γ), which allowed direct comparison of the results from different membranes and donors.

**RNAse Protection Assay**—Sets of human chemokine and cytokine template RPA probes (Pharmingen, San Diego, CA), containing mixtures of linearized plasmids ready for use as templates for in vitro synthesis of antisense RNA, were transcribed into antisense RNA for 6 h at 37 °C with T7 RNA polymerase in the presence of unlabeled ATP, CTP, GTP, and UTP using Ambion (Austin, TX) MAXiscript and ME-GAscript kits. The probes were digested with DNase I (2000 units/ml) at 37 °C for 15 min, extracted with phenol, precipitated with LiCl and isopropanol, and purified by elution from 8 μm polyacrylamide gels and precipitation with ammonium acetate and ethanol. The purified antisense RNA probes were labeled with psoralen-biotin using the Ambion BrightStar Psoralen-Biotin Nonisotopic Labeling Kit. Briefly, 0.5 μg of RNA was incubated for 45 min under 365-nm UV light with 10 μg of sheared salmon sperm DNA for 30 min and hybridization was performed at 68 °C overnight with 1–5 × 10^6 cpm of denatured cDNA probe, as recommended by the manufacturer. The membranes were washed at 68 °C to a final stringency of 0.1× SSC, 0.5% SDS, and subjected to autoradiography. Each spot was quantified by Kodak Digital Science Image Station 440CF and Image Analysis Software version 3.0. The results were then normalized based on hybridization to two housekeeping genes (actin and ribosomal protein S9) whose expression did not change upon stimulation (with the exception of IFN-γ, for which only S9 was used, because expression of actin mRNA was enhanced by IFN-γ), which allowed direct comparison of the results from different membranes and donors.

**RESULTS**

Stimulation of human monocytes with PGN, *S. aureus*, or LPS resulted in activation of over 120 genes, out of 600 genes tested (Fig. 1). Quantification of the amount of mRNA in the induced samples revealed that 12–15 genes were very strongly induced (10–50-fold increase over the control), 20–25 genes showed intermediate induction (5–10-fold increase), and the remaining 80–90 genes showed lower induction (2–5-fold increase) (Fig. 2).

Chemokine genes were the two most highly induced genes by all membranes, cross-linked with UV, and air-dried. The protected RNA fragments were then detected with streptavidin-alkaline phosphatase and a BrightStar BioDetect nonisotopic detection kit (Ambion), as recommended by the manufacturer, and a Kodak Digital Science Image Station 440CF. Hybridization of cellular RNA with specific RNA probes protects labeled RNA probes from RNAse digestion and yields protected probe fragments, which are 20–30 nucleotides shorter than the unprotected probes. RNA molecular weight markers of 100, 200, 300, 400, 500, 750, and 1000 bases were transcribed with T7 RNA polymerase from Ambion RNA Century Marker Plus templates. The quantitative results were normalized based on two housekeeping genes (L32 and GAPDH). This allowed direct comparison of the results from different membranes and donors, including comparing the amounts of chemokine RNA with cytokine RNA.

**Enzyme-linked Immunosorbent Assay**—Chemokine and cytokine concentrations in culture supernatants were determined by capture enzyme-linked immunosorbent assays with monoclonal anti-human IL-8, MIP-1α, MIP-1β, IL-1β (from R&D Systems Inc., Minneapolis, MN), or MIP-1 (from Pharmingen) capture antibodies, and polyclonal biotinylated detection antibodies (from R&D Systems or Pharmingen, respectively), as recommended by the manufacturers. TNF-α concentrations were determined using the L929 cytotoxicity assay described previously (22).
Fig. 2. Chemokines are the highest induced genes in PGN-, S. aureus-, and LPS-, but not in IFN-γ-stimulated monocytes. Expression of the top 40 genes on Atlas cDNA arrays (prepared as in Fig. 1) was quantified and normalized based on two housekeeping genes. For IFN-γ, 20 of the same genes shown for bacterial stimulants are followed by the top 20 genes that were induced by IFN-γ. The results are means from three different donors and three lots of membranes (two donors and two membranes for IFN-γ). The S.E. values were less than 15% and are not shown.
three bacterial stimulants (Fig. 2). Genes for chemokines IL-8, MIP-1α, MIP-2α, MIP-1β, and MCP-1 were the five highest activated proinflammatory genes induced by PGN (higher than the cytokine genes). Genes for chemokines IL-8 and MIP-1α were also the two highest induced proinflammatory genes by S. aureus cells and LPS, and genes for other chemokines (MIP-2α, MIP-1β, and MCP-1) were also induced as high or higher than the cytokine genes (TNF-α, IL-1, and IL-6, which are usually assumed to be the most highly induced proinflammatory mediators by bacterial products; see Refs. 1–7).

mRNA for a zinc finger protein, DB-1, was also one of the highest induced genes by all three bacterial stimulants. Other genes highly induced by bacterial stimulants included HLA class I, MRP-14, MRP-8, thymosin β-10, and Y-box-binding protein-1 (Fig. 2).

The gene expression patterns induced by PGN, S. aureus, and LPS were similar, but not identical, i.e. each stimulus induced a unique gene expression pattern that was reproducible in separate experiments on different donors. For example, the TNF-α gene was induced less by PGN than by LPS and S. aureus. DB-1 gene was induced more by LPS than by PGN and S. aureus, and the MCP-1 gene was induced more by PGN and LPS than by S. aureus (Figs. 1 and 2).

A nonbacterial monocyte activator, IFN-γ, induced more than 200 genes in human monocytes. However, the gene expression pattern induced by IFN-γ was completely different from the gene expression pattern induced by the bacterial stimulants (Figs. 1 and 2). Out of the top five chemokine genes that were most strongly induced by the bacterial stimuli, IFN-γ strongly induced expression of only MCP-1, whereas other chemokine genes (IL-8, MIP-1α, MIP-2α, and MIP-1β) were not induced or were much less induced by IFN-γ. These results are consistent with the previously reported high induction of MCP-1, but not IL-8, by IFN-γ in human macrophages (23).

Similarly, cytokine genes (TNF-α and IL-6) or the gene for DB-1, which were highly induced by the bacterial stimulants, were also not induced or were much less induced by IFN-γ. Besides the high induction of the MCP-1 gene, IFN-γ also highly induced genes for two other chemokines (MIG and IP-10) and several other genes (natural killer cell-enhancing factor B (NKEFB), tyrosine kinase receptor related to TRK (TKTR), IL-17, MARCKS-related protein (MacMARCKS), protein C inhibitor (PCI), guanine nucleotide-binding protein α-13 (GNA13), calcium/calmodulin-dependent protein kinase IV (CAMK4), etc.), which were not induced or were much less induced by the bacterial stimulants (Figs. 1 and 2).

To verify the results obtained with the cDNA array, we used the RNase protection assay to quantify the amounts of chemokine and cytokine mRNA induced by PGN, S. aureus, and LPS. This method also showed that the amount of chemokine mRNA induced by these stimulants was higher than the amount of cytokine mRNA, and again IL-8, MIP-1α, MIP-1β, and MCP-1 were the main chemokines highly induced by all three bacterial stimulants (Fig. 3). Other chemokines (RANTES, lymphotactin, IP-10, and I-309) were not significantly induced by any of these stimuli (Fig. 3).

Out of the cytokines, IL-1β mRNA was the highest induced by all three stimuli (Fig. 3). PGN also induced a moderate increase of IL-1α, TNF-α, IL-1Ra, and IL-6 mRNA and a small increase of granulocyte colony-stimulating factor mRNA, whereas S. aureus also induced a high increase of TNF-α mRNA, a moderate increase of IL-1Ra and IL-1α mRNA, and a lower increase of IL-6 and granulocyte colony-stimulating factor mRNA (Fig. 3). LPS also induced a high increase in TNF-α mRNA; a moderate increase in IL-1α, IL-1Ra, and IL-6 mRNA; and a lower increase in granulocyte colony-stimulating factor mRNA (Fig. 3). Neither of the bacterial stimulants induced significant increase in IL-12 p35, IL-12 p40, and IFN-γ mRNA (Fig. 3).

We and others have previously shown that human monocytes stimulated with PGN, S. aureus, and LPS secrete several cytokines, including IL-1, TNF-α, and IL-6 (1–7, 19, 22, 25), and monocytes stimulated with LPS or bacteria produce chemokines (2, 24, 26–28). However, chemokine induction by PGN has never been studied. Therefore, the next experiments were done to determine if PGN induces chemokine secretion and also to compare in the same culture supernatants the amounts of chemokines and cytokines induced by the three bacterial stimulants.

All three stimulants (PGN, S. aureus, and LPS) induced secretion of very large amounts of IL-8 and MCP-1 (Fig. 4). They also induced several hundred- to several thousand-fold increases in secretion of MIP-1α and MIP-1β, but the total amount of MIP-1α and MIP-1β released was 5–10 times lower than IL-8 and MCP-1 (Fig. 4). All three bacterial stimulants were equally potent in inducing IL-8 secretion, whereas PGN was the strongest inducer of MCP-1, and LPS was the strongest inducer of MIP-1α and MIP-1β. The total amounts of cytokines TNF-α and IL-1β induced by S. aureus and LPS were 5–10 times lower than the amount of IL-8 and MCP-1, and the amounts induced by PGN were approximately 10 times lower than the amounts induced by S. aureus and LPS (Fig. 4). Thus, PGN induced larger amounts of all four chemokines than cytokines, and S. aureus and LPS induced larger amounts of IL-8 and MCP-1, and similar amounts of MIP-1α, MIP-1β, TNF-α, and IL-1β. These results confirm the gene expression data and show that also at the protein secretion level, IL-8 is the highest induced chemokine by all three bacterial stimuli.

Chemokines were most likely induced directly by bacteria and bacterial products (rather than indirectly by bacterially induced TNF-α and IL-1), because the kinetics of induction of most chemokine genes and chemokine secretion (except MCP-1) was very rapid and usually preceded or paralleled the cytokine induction. Moreover, the level of induction of chemokine mRNA and protein was higher than the level of cytokine mRNA and protein, and the ability of various stimuli to induce chemokines did not correlate with their ability to induce cytokines. For example, PGN induced low levels of TNF-α mRNA and protein; yet, it induced as high levels of chemokine mRNA and protein as the other stimuli.

Slower kinetics of induction of MCP-1 is consistent with its newly discovered anti-inflammatory properties (24). MCP-1, in addition to its role in chemotaxis, also down-regulates production of proinflammatory cytokines (24), which would be beneficial at the later, rather than early, stages of bacterially-induced inflammatory process.

Essentially similar results were obtained with human monocytic THP-1 cells stably transfected with CD14; i.e. genes for chemokines showed the highest expression in PGN-, S. aureus-, and LPS-stimulated cells using cDNA arrays (not shown) and an RNase protection assay (Fig. 5), although few differences from monocytes were noted, such as lower expression of IL-8.
mRNA, higher induction of TNF-α than IL-1β mRNA, relatively high expression of RANTES mRNA, and lower responsiveness to LPS. These results further confirm high induction of chemokine mRNA in monocytes and demonstrate that for most chemokines and cytokines, THP-1 cells are a valid model for studying the mechanism of human monocyte responsiveness to bacterial stimuli.

DISCUSSION

Our data demonstrate that bacterial products activate more than 120 genes in human monocytes and that chemokine genes are the main proinflammatory genes activated by cell wall components of both Gram-positive and Gram-negative bacteria. By contrast, a nonbacterial monocyte activator, IFN-γ, induces expression of totally different genes. Secretion of che-
mokine proteins is also very highly induced by bacterial stimulants. The most strongly induced chemokine is IL-8. IL-8 and MIP-2α (which is also strongly induced) belong to the CXC chemokine subfamily, are chemotactic primarily for neutrophils (and also for T cells, NK cells, endothelial cells, basophils, and eosinophils) and stimulate neutrophil degranulation, adhesion, and microbicidal activity (7, 26–29). Other chemokines also strongly induced by bacterial cell wall components are MIP-1α, MIP-1β, and MCP-1. They belong to the CC chemokine subfamily, are chemotactic primarily for monocytes and T cells (and also for NK cells, dendritic cells, and basophils), and activate T cells and macrophages (7, 26–29).

These results are consistent with highly inflammatory and pyogenic nature of bacterial infections and with extensive infiltration of the sites of bacterial infections with polymorphonuclear and mononuclear cells (30). Together with other studies (31, 32), they suggest that chemokines are the major factors recruiting all of these cells to the sites of infection. These results may also have implications for other pathophysiologic changes seen in bacterial infections, since chemokines have diverse effects on various other systems in the body, including hemopoiesis, angiogenesis, and central nervous system (26, 27, 29, 33).

Another gene highly induced by the bacterial stimuli was DB-1, which codes for a ubiquitous zinc finger protein that binds to the IL-3 promotor (34). However, because IL-3 is normally produced by T cells, and because we did not detect any increase in the expression of IL-3 mRNA in bacterially stimulated monocytes, DB-1 must have another as yet unidentified function in monocytes.

In general, the genes activated by all three bacterial stimulants were similar, which is not surprising, because they all activate monocytes through CD14 and TLR2 receptors (1, 2, 9–17). However, the specific gene expression patterns induced by each stimulus were different, which may be due to several differences in the function of CD14 as the PGN and LPS receptor (11–17). By
contrast, IFN-γ, which activates cells through a different (Jak/ signal transducers and activators of transcription-mediated) signal transduction pathway (36), induces activation of a totally different gene pattern (with the exception of one gene, MCP-1, which is strongly induced by both IFN-γ and bacterial stimulants).

PGN was a much weaker inducer of TNF-α mRNA expression and protein secretion than S. aureus and LPS. This is consistent with the lack of toxicity of PGN in animals. For example, in galactosamine-treated mice, PGN is not toxic at 50 μg/mouse, whereas for LPS, LD₅₀ = 4 ng/mouse and LD₉₀ = 100 ng/mouse, or in adrenalectomized mice, LD₅₀ for PGN is >300 μg/mouse and for LPS is 0.1 μg/mouse (37). The high induction of TNF-α by the whole S. aureus cells, but not by PGN, is also consistent with the shock-inducing capacity of S. aureus infections (2–4), but not of isolated PGN alone (2, 8), and is probably due to synergistic action of PGN and lipoteichoic acid (8). These results are consistent with the notion that TNF-α, but not IL-1β, is the main determinant of toxicity (3–7).

In summary, chemokine genes show the highest induction

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**Fig. 5.** Kinetics of expression of chemokine and cytokine mRNA in PGN-, S. aureus-, and LPS-stimulated THP-1/CD14 monocytic cells quantified by RNase protection assay. Human THP-1/CD14 stable transfectants were stimulated, and RNase protection assay was performed as in Fig. 3. Representative blots are shown on the left. The graphs show means from three experiments, normalized for each membrane based on two housekeeping genes. The S.E. values were less than 10% and are not shown.

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² R. Dziarski and J. T. Ulrich, unpublished observations.
out of 120 genes induced in human monocytes by PGN, LPS, and bacterial cells. By contrast, expression of a different gene pattern is induced by a nonbacterial stimulus, IFN-γ.

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