Chemokine Secretion by Human Polymorphonuclear Granulocytes after Stimulation with Mycobacterium tuberculosis and Lipoarabinomannan

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Received 6 March 1997/Returned for modification 2 June 1997/Accepted 31 August 1997

Macrophages (MAC) and polymorphonuclear granulocytes (PNG) are professional phagocytes which perform essential functions in antibacterial defense. The intracellular bacterium Mycobacterium tuberculosis persists and replicates in resting macrophages. Although it is generally assumed that activated MAC are central to protection against M. tuberculosis, PNG may also contribute to defense. We wondered whether PNG produce proinflammatory chemokines after stimulation by M. tuberculosis or its major cell wall component, lipoarabinomannan (LAM). In this study, we showed that M. tuberculosis- and LAM-activated human PNG secrete the leukocyte attractant interleukin-8 (IL-8) and the PNG-specific chemokine GRO-α in a dose-dependent manner. Treatment of PNG with the leukotriene-B4 inhibitor MK-886 prior to stimulation with M. tuberculosis or LAM partially blocked IL-8 and GRO-α induction, suggesting involvement of the 5-lipoxygenase pathway in the secretion of these chemokines. We conclude that PNG contribute to early resistance to M. tuberculosis via chemokine secretion.

Tuberculosis is a major health threat to mankind which is responsible for almost 3 million deaths per year. The responsible pathogen, Mycobacterium tuberculosis, is an intracellular bacterium that survives and replicates in resting macrophages (8). Convincing evidence that activated macrophages (MAC) are responsible for control of M. tuberculosis has been presented (8). The highly complex cell wall of M. tuberculosis is rich in lipoglycans, and lipoarabinomannan (LAM), an abundant component of the mycobacterial cell wall, is a potent immunomodulator which acts on T cells and MAC (9). Polymorphonuclear granulocytes (PNG) are the first cells which, in response to microbial invasion, migrate from the blood into tissue sites, where they participate in the early inflammatory response. Their principal role in inflammation and host defense has long been thought to be restricted to phagocytosis and bacterial killing. Various stimuli activate the generation of reactive oxygen intermediates and the release of lytic enzymes with potent antimicrobial potential (4). PNG are short-lived and often regarded as terminally differentiated cells that are devoid of transcriptional activity and capable of performing little, if any, protein synthesis (4). However, in the past few years, in vitro studies have revealed that PNG produce a variety of proteins upon proper stimulation. Major histocompatibility complex class II expression after treatment with granulocyte-macrophage colony-stimulating factor was demonstrated (4), and a number of cytokines, including tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), and transforming growth factor β (4), are actively produced after challenge with various stimuli. These findings suggest that PNG play an active role in inflammatory and immune responses. Although PNG have been implicated in resistance to tubercle bacilli (3), numerous results to the contrary have been reported (5).

Chemokines are potent leukocyte attractants which can be divided into two major groups according to their characteristic sequence motifs and their target cells (1). Those of the CXC type primarily act on PNG, whereas CC-type chemokines preferentially act on monocytes (MO)/MAC. IL-8 and GRO-α not only are both CXC-type chemokines; they also show marked sequence homology. Both chemokines are expressed by MO and PNG upon appropriate stimulation, and receptors for GRO-α and IL-8 have been identified on PNG and on natural killer (NK) cells (10). IL-8 and GRO-α share many biological activities, including induction of chemotaxis, exocytosis, and respiratory burst. In addition, IL-8, but not GRO-α, upregulates complement receptors 1 and 3 (10). Leukotrienes (LT) are proinflammatory lipid compounds, derived from arachidonic acid metabolism via the 5-lipoxygenase pathway (14), which are produced by hematologic cell types. LTβ is a potent chemotactic and activating agent for leukocytes. It stimulates platelet aggregation, release of lysosomal enzymes, and generation of O2 metabolites by PNG (14). LTβ augments IL-1 and TNF-α production of MO/MAC (7, 14). 5-Lipoxygenase is a dual-function enzyme that generates the epoxide LTA₄, which is central to LT biosynthesis. Within PNG, LTA₄ is converted to the chemoattractant LTB₄ by LTA₄ hydrolase (14). LTB₄ inhibitors, such as MK-886, inhibit LT biosynthesis in intact cells (11, 16).

In this study, we have demonstrated that M. tuberculosis and its major cell wall component, LAM, induce IL-8 and GRO-α in human PNG at the mRNA and protein levels. Inhibition of LTβ synthesis partially blocked chemokine secretion, revealing a fundamental role of LT in inflammation and PNG activation. We suggest that PNG contribute to the early host response against M. tuberculosis by increasing local inflammation and recruiting professional phagocytes.

MATERIALS AND METHODS

Microorganisms and LAM. M. tuberculosis H37Rv was a generous gift of J. K. Seidel (Forschungsinstitut Borstel, Borstel, Germany). Mycobacteria were grown in Dubos broth (Difco, Detroit, Mich.), and bacterial counts were evaluated by determining CFU on Middlebrook 7H10 agar (Difco) cultures incubated at 37°C for 3 weeks. LAM was a generous gift of P. J. Brennan (Department of Micro-

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biology, Colorado State University, Fort Collins) under National Institute of Allergy and Infectious Diseases contract NOI AI-52582. LAM was isolated from a rapidly growing Mycobacterium species, and the endotoxin concentration was 4.4 ng of endotoxin per mg of LAM as determined by Limulus amoeboocyte assay (Sigma).

Preparation of human PNG. Leukocyte buffy coats obtained from healthy donors (Blutbank, Ulm, Germany) were separated over Ficoll-Hypaque 1.077 (Sigma).

Culture supernatants of both stimulated and unstimulated cells were lysed by the addition of guanidine isothiocyanate. The following RNA extraction was performed with the RNeasy kit (Qiagen, Hilden, Germany). Total cellular RNA was used for reverse transcription, both stimulated and unstimulated cells were lysed by the addition of LAM (0.025, 0.25, and 2.5 \( \mu \)g/ml) as indicated in the figures. Supernatants were harvested, and IL-8 was measured by ELISA. nil, unstimulated serum control. Data represent the means of values for duplicate samples (standard deviations, <10%) from one representative experiment of three with comparable results. Viability of stimulating \( M. \) tuberculosis cells was revealed by plating serial dilutions on Middlebrook agar plates.

FIG. 1. \( M. \) tuberculosis- and LAM-induced release of IL-8 by human PNG. (A) PNG (10\(^6\)) were stimulated with various numbers of \( M. \) tuberculosis cells (10\(^6\), 10\(^5\), or 10\(^3\)) and cultured for 4 h. Supernatants were harvested, and IL-8 was measured by ELISA. (B) PNG (10\(^6\)) were stimulated with various concentrations of LAM (2.5 \( \mu \)g/ml, 0.25 \( \mu \)g/ml, or 0.025 \( \mu \)g/ml). Supernatants were harvested, and IL-8 was measured by ELISA. nil, unstimulated serum control. Data represent the means of values for duplicate samples (standard deviations, <10%) from one representative experiment of three with comparable results. Viability of stimulating \( M. \) tuberculosis cells was revealed by plating serial dilutions on Middlebrook agar plates.

RESULTS

\( M. \) tuberculosis and LAM stimulate IL-8 and GRO-\( \alpha \) secretion by PNG. Freshly isolated PNG (10\(^6\)) were treated with different numbers of \( M. \) tuberculosis (10\(^6\) to 10\(^3\)) cells for 4 h. Culture supernatants were collected, and IL-8 concentrations were determined by ELISA. As shown in Fig. 1A, \( M. \) tuberculosis-stimulated human PNG released the leukocyte attractant IL-8. This release of IL-8 critically depended on the ratio of PNG to \( M. \) tuberculosis cells. At a ratio of 1:1, PNG responded with a 10- to 50-fold increase in IL-8 release. At lower mycobacterial numbers, the amount of IL-8 decreased, reaching background levels at a ratio of 1,000:1. We were interested in determining whether the major mycobacterial cell wall component of \( M. \) tuberculosis, LAM, stimulated PNG. LAM was shown to possess IL-8-inducing capacity, causing a 6- to 10-fold increase in IL-8 (Fig. 1B). The existence of an IL-8-inducing capacity of \( M. \) tuberculosis- or LAM-stimulated human PNG was supported by the finding of an increase in mRNA production as revealed by semiquantitative PCR (Fig. 2). RT-PCR data corresponded with those from the protein secretion studies. GRO-\( \alpha \), a chemokine which recruits and activates human PNG, was also induced after PNG stimulation with \( M. \) tuberculosis and LAM (Fig. 3). Mycobacterial treatment stimulated uptake to a 12-fold increase of GRO-\( \alpha \) in a concentration-dependent manner (Fig. 3A). Similarly, stimulation of PNG by LAM induced a concentration-dependent release of GRO-\( \alpha \) (Fig. 3B).
3B). Stimulation of human PNG with the same concentration (2.5 μg/ml) of lipo polysaccharide (LPS) led to even a higher level of chemokine production (15,000 pg/ml for IL-8 and 550 pg/ml for Gro-α). In contrast, stimulation with 10 pg of LPS/ml did not cause PNG-mediated chemokine secretion (data not shown). These data exclude the possibility that LPS contamination is responsible for the effects observed with LAM. Evaluation of Gro-α induction by semiquantitative PCR revealed an increase in Gro-α PCR fragment production (Fig. 2). PCR data (Fig. 2) correspond to the increasing protein secretion determined by ELISA (Fig. 1), demonstrating increased PCR cDNA fragment production for IL-8 and Gro-α after stimulation with M. tuberculosis or LAM. Differences in the appearance of the brightness of the PCR fragments seem to be caused by variations in donor cells. LAM was preincubated with polymyxin B (5 μg/ml) to neutralize potentially contaminating endotoxin and then added to the cells. Polymyxin B treatment did not affect chemokine production (data not shown).

Interference of MK-886 with M. tuberculosis- and LAM-induced chemokine secretion by PNG. The LTB4 inhibitor MK-886 blocks the activity of 5-lipoxygenase-activating protein (11, 16). Consequently, cells fail to produce any LT because LTB4 is unavailable as a substrate. Treatment of PNG with the leukotriene-B4 inhibitor MK-886 prior to stimulation with M. tuberculosis or LAM partially blocked IL-8 and Gro-α secretion (Table 1). Inhibition was concentration dependent, with maximal inhibitions of 75% for Gro-α and 60% for IL-8 at 50 μg of MK-886/ml (both with M. tuberculosis). The reduction of Gro-α was more pronounced than that of IL-8. In the presence of 5 or 50 μg of inhibitor/ml, cell survival was 83 or 70%, respectively, whereas higher concentrations caused a more dramatic loss of cell viability, as assessed by Trypan blue exclusion (data not shown). Thus, even taking the unspecific cytotoxicity of MK-886 into account, specific inhibition of chemokine secretion by MK-886 still occurred.

**DISCUSSION**

In this study, we have shown that PNG respond to M. tuberculosis and its major cell wall component, LAM, by secreting the chemokines IL-8 and Gro-α in vitro. Our data suggest the contribution of chemokines produced during the host response to tuberculosis. Not only M. tuberculosis but, more importantly, LAM stimulated production of the chemokines IL-8 and Gro-α by PNG. Consistent with findings obtained in other systems (3, 4, 13), our data emphasize that PNG are actively synthesizing cells and suggest that these cells participate in the early host response to M. tuberculosis. Similarly, activation of human PNG by M. tuberculosis sulfolipids has been reported to stimulate superoxide generation (18). We assume that two mechanisms underlie chemokine induction in PNG, the first one being dependent on, and the second one being independent of, phagocytosis. Consistent with this assumption, PNG produce IL-8 after phagocytosis of heat-killed yeast (2) and after stimulation by LPS at 100 ng/ml (6). We also detected endotoxin (2.5 μg/ml)-induced chemokine production by PNG but found no chemokine induction with low doses of LPS (10 pg/ml). Involvement of contaminating LPS in LAM-induced chemokine secretion in our experiments, therefore, can be excluded. LAM and LPS possibly activate PNG through the same receptor, since cytokine induction in the human monocytoid cell line THP-1 by LAM and LPS was inhibitable by a monoclonal antibody to CD14 (20). However, they differ in that LAM appears to require an additional signalling receptor component restricted to hemopoietic cells (15). Thus, common receptor components for LAM and LPS may be expressed on PNG, including the CD14 signalling system.

IL-8 and Gro-α are CXC-type chemokines which are chemotactic for PNG but not MO/MAC. Gro-α activates PNG and NK cells (1, 10), whereas IL-8 is chemotactic for T lymphocytes in addition, although at a later time point (17). M. tuberculosis-infected MAC synthesize various proinflammatory agents.

**TABLE 1. Inhibition of chemokine release by the LTB4 inhibitor MK-886**

<table>
<thead>
<tr>
<th>Inducer (inhibitor)</th>
<th>Chemokine concn, pg/ml (% inhibition)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>IL-8</td>
</tr>
<tr>
<td>M. tuberculosis (none)</td>
<td>11,900 (0%)</td>
</tr>
<tr>
<td>M. tuberculosis (MK-886, 5 μg/ml)</td>
<td>8,120 (30%)</td>
</tr>
<tr>
<td>M. tuberculosis (MK-886, 50 μg/ml)</td>
<td>4,820 (60%)</td>
</tr>
<tr>
<td>LAM (none)</td>
<td>2,050 (75%)</td>
</tr>
<tr>
<td>LAM (MK-886, 5 μg/ml)</td>
<td>1,130 (45%)</td>
</tr>
<tr>
<td>LAM (MK-886, 50 μg/ml)</td>
<td>1,230 (40%)</td>
</tr>
</tbody>
</table>

*PNG were treated with increasing concentrations of the LTB4 inhibitor MK-886 (5 or 50 μg/ml) for 5 min; cells were incubated with M. tuberculosis (10⁶ cells) or stimulated by LAM (2.5 μg/ml) for 4 h. Supernatants were collected and assayed for IL-8 and Gro-α by ELISA; parentheses denote the percentage of MK-886-mediated inhibition. Data represent the means of values for duplicate specimens (standard deviations, <10%) for one representative experiment of three with comparable results.

*Unless otherwise noted, P < 0.05 (Student’s t test).

*P < 0.01 (Student’s t test).
cytokines, including TNF-α and IL-8 (19), and present mycobacterial antigens to T lymphocytes (8). Upon stimulation by an appropriate antigen, T cells produce an array of cytokines, including gamma interferon (IFN-γ) and IL-2. IFN-γ is the major MAC-activating cytokine which causes NO production and subsequent inactivation of *M. tuberculosis*, at least in the experimental mouse model (8). Although the mechanisms underlying tuberculosis control in humans are still enigmatic, evidence suggesting that similar mechanisms are involved is accumulating. In particular, activation of inducible nitric oxide synthase has been detected in human alveolar MAC from tuberculosis patients (12). Furthermore, successful resolution of tuberculosis in an immunocompromised patient was achieved by adjunctive therapy with IFN-γ and granulocyte colony-stimulating factor (13). The latter cytokine is known to act on PNG.

LT are important inflammatory molecules with potent chemotactic activity for PNG (14). Moreover, they increase production of the monokines TNF-α and IL-1β and stimulate exocytosis in MAC (7, 14). Because PNG activation results in LTB4 synthesis, we tried to block eicosanoid production by MK-886 interfered with chemokine release by LAM- or *M. tuberculosis*-treated PNG, with the effects on LTβ4 depending on the inhibitor concentration. Our results are not only in agreement with the involvement of LTβ4 in monokine production (14); they also emphasize the importance of LT in inflammation.

**ACKNOWLEDGMENTS**

This work was funded by the BMBF Verbundprojekt “Mykobakterielle Infektionen.”

We thank Christoph Ladel (Department of Immunology, Ulm University) for helpful discussions and Patrick Brennan (Colorado State University, Fort Collins) for generously providing the lipoarabinomannan.

**REFERENCES**


Editor: R. E. McCallum