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Regulation of Chemokine Gene Expression in Human Peripheral Blood Neutrophils Phagocytosing Microbial Pathogens

Mohamed Hachicha,* Palaniswami Rathanaswami,* Paul H. Naccache,† and Shaun R. McColl2*

Production of chemokines (chemotactic cytokines) by neutrophils is likely to be important in the regulation of inflammation and the control of infection. In this study we show that exposure of human neutrophils to various microbial pathogens leads to the production of both macrophage inflammatory protein 1α (MIP-1α) and IL-8. The bacterial microbes, Salmonella typhimurium and Pseudomonas aeruginosa, and Staphylococcus aureus all strongly induced both IL-8 and MIP-1α secretion, whereas Streptococcus pneumoniae, Staphylococcus epidermidis, and the opportunistic yeast Candida albicans were less potent. Saccharomyces cerevisiae and zymosan both induced IL-8 secretion but failed to stimulate that of MIP-1α. Coincubation of neutrophils with the proinflammatory cytokine TNF-α and the micro-organisms also led to differential expression of MIP-1α and IL-8. Significant enhancement of the induction of both MIP-1α and IL-8 by S. typhimurium, P. aeruginosa, and S. pneumoniae as well as by C. albicans was observed. In contrast, while IL-8 production in response to S. cerevisiae and zymosan was enhanced in the presence of TNF-α, no MIP-1α was produced. These combined results indicate that while neutrophils exposed to some micro-organisms alone or in the presence of inflammatory cytokines such as TNF-α will produce both MIP-1α and IL-8, resulting in generation of signals for the recruitment of mononuclear leukocytes and neutrophils, respectively, certain types of micro-organisms can skew this response toward synthesis of IL-8. The Journal of Immunology, 1998, 160: 449–454.
that this cell population may be capable of directing its own recruitment to sites of inflammation and infection and thus autoamplify acute inflammatory responses to infection. In addition, recent studies show that neutrophils also produce the C-C chemokines MIP-1α and MIP-1β in response to TNF-α and LPS (19, 20), which theoretically gives them the capacity to emit signals to recruit mononuclear cells. However, our recent studies show that MIP-1α synthesis is inhibited by neutrophils phagocytosing the inflammatory microcrystals monosodium urate (MSU) and calcium pyrophosphate dihydrate (CPPD) (11), whereas under the same conditions IL-8 synthesis is enhanced. The present study was undertaken to determine whether the inhibition of MIP-1α production by MSU and CPPD aligned with enhancement of IL-8 secretion is a general phenomenon in neutrophils undergoing phagocytosis. Since a major function of the neutrophil in the innate immune system is phagocytosis of microbial pathogens during infection, we have investigated the regulation of MIP-1α and IL-8 expression by neutrophils during phagocytosis of microbial pathogens.

Materials and Methods

Materials

TNF-α was a gift from Knoll Pharmaceuticals (Whippany, NJ). TNF-α stock was stored at −80°C in PBS containing 0.01% BSA. Zymosan was obtained from Sigma/Aldrich (Castle Hill, Australia). HBSS and RPMI medium 1640 were purchased from Life Technologies (Mulgrave, Australia). Ficoll-Paque and Dextran T-500 were purchased from AMRAD/Pharmacia (Wantirna, Australia). HyClone FCS was purchased from Professional Diagnostics (Edmonton, Canada). All the above reagents contained <5 pg/ml of endotoxin as determined by the amebocyte lysate assay (Bio-Whittaker, Walkersville, MD). Staphylococcus aureus, Salmonella typhimurium, Pseudomonas aeruginosa, Streptococcus pneumoniae, and Staphylococcus epidermidis were obtained from stocks within the Department of Microbiology and Immunology at the University of Adelaide. Candida albicans and Saccharomyces cerevisiae were purchased from American Type Culture Collection (Rockville, MD). Hybond-N membranes, [γ-32P]ATP, and [α-32P]dCTP were purchased from Amersham Canada (Oakville, Canada). All other reagents used in this study were of molecular biologic grade and were obtained from Sigma/Aldrich.

Preparation of opsonized zymosan and opsonized micro-organisms

The bacteria and yeast were harvested, resuspended in 20 ml of sterile RPMI 1640, and incubated for 1 h at 100°C. The bacteria were washed twice with sterile RPMI 1640 and counted. The pellet was resuspended in 20 ml of nondecomplemented human serum and incubated for 1 h at 37°C, and the bacteria were washed twice with RPMI 1640. The pellet was then resuspended in a sterile solution of RPMI 1640 at a concentration of 10^10 microbes/ml. The same procedure was followed to opsonize zymosan at a final concentration of 30 ng/ml.

Isolation of human PMNL

Whole blood was obtained by venipuncture and collected into tubes containing heparin; following dextran sedimentation, neutrophils were purified by centrifugation on Ficoll-Paque cushions (11, 21). Erythrocytes were removed by hypotonic lysis, and the cells were resuspended in RPMI 1640 supplemented with 1% FCS at a final concentration of 10^7 cells/ml. One-milliliter aliquots were used for all experimental points except those involved in the variation of total RNA for Northern analysis, in which case 4-ml aliquots were used. Cell viability, as determined by trypan blue exclusion, was >98%, and the percentage of neutrophils in the cell preparations used in this study exceeded 97%. On average, the other 3% comprised other granulocytes (2%), lymphocytes (0.8%), and monocytes (at most 0.2%). Only neutrophil preparations containing <2 × 10^5 monocytes/10^7 cells were used in this study. Detectable levels of IL-8 and MIP-1α were only observed when 5 × 10^5 purified monocytes or more were incubated in the presence of TNF-α or phagocytic agonists. The entire separation was conducted under sterile conditions at room temperature.

Isolation of cytoplasmic RNA and Northern blot analysis

After activating cells with the desired stimuli, total RNA was prepared by the RNAzol method as recommended by the supplier, and Northern blots were performed as previously described (6, 11). The cDNA probes used in this study were radioabeled with [α-32P]dCTP using the random primers DNA labeling system. The human MIP-1α cDNA was isolated using PCR with an antisense primer made against nucleotides 283 to 236 of the published LD78 sequence and a sense primer comprising nucleotides 19 to 3 (11). The IL-8 probe used in this study was a 244-bp PstI/EcoRI dDNA fragment representing the coding region of the IL-8 cDNA from nucleotides 49 to 293 (11). To confirm equal loading of RNA, the membranes were rehybridized with a synthetic oligonucleotide for 28S ribosomal RNA (data not shown) as described previously (22).

ELISA for IL-8 and MIP-1α

Human neutrophils were isolated and resuspended at 10^7 cells/ml in 4-ml sterile tubes and treated as described in the figure legends. The supernatants were collected and analyzed for chemokine content. In experiments involving the assessment of intracellular chemokine content, the cell pellets were washed three times in ice-cold sterile PBS and lysed by three consecutive freeze-thaw cycles. Levels of IL-8 and MIP-1α were assessed using ELISA kits purchased from R&D Systems, Inc. (Minneapolis, MN).

Statistical analysis

Numerical values in the figures are the levels of immunoreactive IL-8 or MIP-1α per 10^7 neutrophils (mean ± SEM). The data for each group were analyzed as a repeated measures analysis of variance with an unstructured covariance matrix. Statistical significance was considered to be achieved at p < 0.05.

Results

Expression of MIP-1α and IL-8 in neutrophils

To determine the basic kinetics of chemokine production by neutrophils in response to microbial pathogens, the cells were incubated with diluent, TNF-α (100 ng/ml), opsonized zymosan (1 mg/ml), or opsonized heat-killed S. aureus (10^9, bacteria/neutrophil) at 37°C for increasing periods of time. The concentration of each of these agonists was chosen from the results of previous studies (4, 6, 8, 9, 23) or from pilot experiments (not shown). A time-dependent release of MIP-1α and IL-8 was observed in response to TNF-α (Fig. 1). Heat-killed opsonized S. aureus also led to a time-dependent release of both MIP-1α and IL-8. In contrast, opsonized zymosan stimulated the production of IL-8 but not MIP-1α. Opsonization enhanced the efficiency of stimulation of chemokine production, increasing both the rate and the amount of synthesis (data not shown).

We next examined the effects of a range of micro-organisms on the production of MIP-1α and IL-8 by neutrophils. Neutrophils were therefore incubated with heat-killed opsonized bacterial micro-organisms at a ratio of 10:1 (micro-organisms:neutrophils) at 37°C for 24 h. The supernatants were collected and analyzed for IL-8 and MIP-1α content. In terms of IL-8 secretion, the most potent agonists were S. typhimurium, P. aeruginosa, and S. aureus (Table 1). Although stimulating significant IL-8 production above the control values, S. pneumoniae, S. epidermidis, C. albicans, and S. cerevisiae were less potent. As shown in Figure 1, zymosan was also a potent inducer of IL-8 synthesis. As observed with IL-8 secretion, S. typhimurium, P. aeruginosa, and S. aureus were all potent inducers of MIP-1α production compared with basal levels. Moreover, as observed with IL-8 secretion, while stimulating a release of MIP-1α that was significantly greater than the control value, S. pneumoniae, S. epidermidis, and C. albicans were far less potent. In contrast to that observed with respect to IL-8 secretion, S. cerevisiae and zymosan failed to induce detectable secretion of MIP-1α and, in fact, significantly inhibited the basal release of MIP-1α.
Phagocytosis of microbes, cell debris, and other particulate matter by neutrophils is one of the major functions of these cells and may play an important role in the development of adaptive immune responses, possibly through the release of extracellular mediators such as chemokines. We have therefore examined the regulation of chemokine gene expression in neutrophils phagocytosing bacteria and yeast.

The results of the present study show that exposure of human neutrophils to various bacteria generally leads to the production of both MIP-1α and IL-8, although the response depends on the microorganism to which the neutrophil is exposed. The most potent agonist was the Gram-negative micro-organism, S. typhimurium.

Table 1. Effect of various treatments on MIP-1α and IL-8 production by human neutrophils

<table>
<thead>
<tr>
<th></th>
<th>MIP-1α (ng/ml)</th>
<th>IL-8 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.028 ± 0.001</td>
<td>0.69 ± 0.22</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.47 ± 0.10*</td>
<td>5.47 ± 0.32*</td>
</tr>
<tr>
<td>S. aureus</td>
<td>3.23 ± 0.69*</td>
<td>27.54 ± 4.15*</td>
</tr>
<tr>
<td>S. pneumoniae</td>
<td>0.33 ± 0.14*</td>
<td>4.73 ± 1.44*</td>
</tr>
<tr>
<td>S. epidermidis</td>
<td>0.05 ± 0.01*</td>
<td>0.86 ± 0.18*</td>
</tr>
<tr>
<td>S. typhimurium</td>
<td>1.77 ± 0.59*</td>
<td>18.70 ± 5.77*</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>3.45 ± 0.78*</td>
<td>33.50 ± 6.62*</td>
</tr>
<tr>
<td>C. albicans</td>
<td>0.05 ± 0.01*</td>
<td>1.49 ± 0.25*</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>0.012 ± 0.001**</td>
<td>1.39 ± 0.17*</td>
</tr>
<tr>
<td>Zymosan</td>
<td>0.011 ± 0.001**</td>
<td>17.41 ± 3.74*</td>
</tr>
</tbody>
</table>

Neutrophils were incubated with diluent, TNF-α (100 ng/ml), zymosan (300 μg/ml), or heat-killed opsonized microbial pathogens as indicated at a ratio of 10:1 (microbes:neutrophil) in RPMI 1640 supplemented with 1% FCS for 3 h at 37°C. The supernatants were collected and analyzed for the presence of MIP-1α or IL-8 using specific ELISAs. The results are the mean ± SEM of triplicate determinations from three separate experiments.

* Significantly different from control values at p < 0.05.
** Significantly lower than control values at p < 0.05.

Effect of TNF-α and micro-organisms in combination on MIP-1α and IL-8 production

Since neutrophils at sites of inflammation or infection are likely to be exposed simultaneously to several agonists, either soluble or particulate, we examined the effect of coincubation of neutrophils with TNF-α and the micro-organisms on MIP-1α and IL-8 production. Neutrophils were therefore incubated at 37°C with diluent or the micro-organisms, either alone or in combination with TNF-α for 24 h. The supernatants were collected, and the amounts of MIP-1α and IL-8 present were assessed by ELISA. As described above, S. typhimurium, P. aeruginosa, and S. aureus alone were able to induce significant release of IL-8 and MIP-1α, whereas S. pneumoniae and S. epidermidis were far less potent (Fig. 3). When neutrophils were coincubated with TNF-α and the microbial pathogens, differential effects were observed on IL-8 and MIP-1α synthesis. Taking into account the effect of TNF-α alone, IL-8 and MIP-1α secretion was significantly enhanced only in response to S. typhimurium, P. aeruginosa, and S. pneumoniae. The effect of coincubation with TNF-α on the ability of zymosan, C. albicans and S. cerevisiae to induce IL-8 and MIP-1α secretion was also examined (Fig. 4). Incubation in the presence of TNF-α led to greater release of IL-8 in response to C. albicans, zymosan, and S. cerevisiae. No MIP-1α was detected in the supernatants of cells incubated with TNF-α in the presence of zymosan or S. cerevisiae, and the statistical analysis showed that both these agonists significantly inhibited the production of MIP-1α induced by TNF-α.

Discussion

Expression of MIP-1α and IL-8 mRNA in neutrophils

To determine whether the micro-organisms up-regulated MIP-1α and IL-8 expression at the mRNA level, Northern blots were performed. Neutrophils were stimulated under the conditions described above, except that the period of incubation was 3 h rather than 24 h. The cell pellets were collected, total RNA was extracted, and equal amounts of RNA were loaded onto agarose gels for transfer onto nylon filters for Northern analysis (Fig. 2). According to densitometric values (data not shown), all agents except C. albicans and S. cerevisiae induced an accumulation of IL-8 mRNA greater than that of the control. However, while densitometric analysis (data not shown) indicated that S. aureus, S. pneumoniae, S. epidermidis, and P. aeruginosa stimulated an accumulation of IL-8 mRNA greater than the control levels, they were not as potent as zymosan or S. typhimurium. In contrast, all the microbes except C. albicans and S. cerevisiae induced an increase in the level of MIP-1α mRNA at least fivefold greater than the control level. Zymosan also strongly induced accumulation of MIP-1α mRNA.
and *P. aeruginosa*, and *S. aureus* (Gram-positive). Other Gram-positive microbes, *S. pneumoniae* and *S. epidermidis*, were less potent, as was the opportunistic yeast *C. albicans*. While the results of the studies on bacterial microbial pathogens and *C. albicans* showed co-ordinated regulation of MIP-1\(\alpha\) and IL-8 production, differential regulation of the expression of these two chemokines was observed when neutrophils were exposed to zymosan or *S. cerevisiae*. Zymosan and *S. cerevisiae* both induced the production of IL-8. However, in contrast to that observed with all the aforementioned microbial pathogens, neither zymosan nor *S. cerevisiae* induced detectable secretion of MIP-1\(\alpha\). Taken together, these results indicate that neutrophils exposed to bacterial microbial pathogens or *C. albicans* at sites of infection will generally release both MIP-1\(\alpha\) and IL-8, and in view of the known biologic activities of these two chemokines (24–26), this suggests that signals for the recruitment of mononuclear leukocytes and neutrophils, respectively, will be generated. In contrast, agonists such as zymosan, *S. cerevisiae*, and, as previously shown, the inflammatory microcrystals MSU and CPPD (11) fail to induce MIP-1\(\alpha\) production, thereby potentially skewing chemokine production by neutrophils toward IL-8 production, theoretically leading to the enhancement of neutrophil recruitment.

The demonstration that several of the agonists used in this study, the Gram-positive bacteria *S. pneumoniae* and *S. epidermidis* and the opportunistic yeast *C. albicans*, are relatively weak inducers of IL-8 and MIP-1\(\alpha\) production in neutrophils suggests that signals in addition to phagocytosis are required for the induction of high levels of expression of the two chemokine genes. Of relevance, previous studies have demonstrated that LPS is a potent agonist of the induction of both these chemokines by neutrophils (19, 20). The Gram-negative bacteria *S. typhimurium* and *P. aeruginosa* express high levels of LPS and, as shown in the present study, are powerful agonists of both IL-8 and MIP-1\(\alpha\) production by neutrophils. Moreover, *S. aureus* expresses lipoteichoic acid (LTA), which also binds to CD14. Of relevance is a recent study demonstrating the ability of LTA derived from *S. aureus* to induce MIP-1\(\alpha\) production (27). However, several of our data suggest that signals other than CD14 may be involved in the stimulation of chemokine expression. For instance, the two other Gram-positive microbes used in the present study, *S. pneumoniae* and *S. epidermidis*, also possess LTA (28–31) but are weak agonists of IL-8 and MIP-1\(\alpha\) production. Moreover, zymosan (present study) and the inflammatory microcrystals MSU and CPPD (11), which do not contain LPS or LTA, are strong inducers of IL-8 production. Ad-
specific ELISAs. The results are the mean ± SEM of triplicate determinations from three separate experiments. NS, not significantly different from control values; *, significantly different from control values at $p < 0.05$; **, significantly lower than control values at $p < 0.05$.

FIGURE 4. Effect of TNF-α and yeast in combination on MIP-1α and IL-8 protein secretion by neutrophils. Neutrophils were incubated in RPMI 1640 supplemented with 1% FCS for 24 h at 37°C with the indicated combinations of agonists. The same concentrations of agonists were used as indicated in Figure 1 and Table I. The supernatants were collected and analyzed for the presence of MIP-1α or IL-8 using specific ELISAs. The results are the mean ± SEM of triplicate determinations from three separate experiments. NS, not significantly different from control values; *, significantly different from control values at $p < 0.05$; **, significantly lower than control values at $p < 0.05$.

At sites of infection, neutrophils are likely to be exposed to multiple agonists. Activated monocyte/macrophages and neutrophils produce TNF-α, an agent that has been shown to directly stimulate gene expression in neutrophils as well as to prime neutrophils for enhanced responsiveness to other signals (6, 32). In this report we show that coincubation of neutrophils with TNF-α enhances IL-8 and MIP-1α expression in response to S. typhimurium, P. aeruginosa, and S. pneumoniae. IL-8 production in response to zymosan and S. cerevisiae, and IL-8 and MIP-1α production in response to C. albicans, TNF-α failed to enhance IL-8 or MIP-1α expression to any of the other microbial pathogens or zymosan. Previous studies have shown that TNF-α enhances phagocytosis by neutrophils in vitro (33, 34). However, this clearly is not a general phenomenon, since in the present study only coincubation of TNF-α with S. typhimurium, P. aeruginosa, and S. cerevisiae significantly enhanced IL-8 and MIP-1α production. On the other hand, S. cerevisiae and zymosan (which is derived from S. cerevisiae) failed to induce MIP-1α production in the presence or the absence of TNF-α, thereby demonstrating that the induction of MIP-1α by TNF-α is inhibited under these conditions. These observations place zymosan and S. cerevisiae in the same category as the inflammatory microcrystals, MSU and CPPD, both of which not only fail to directly induce MIP-1α production in neutrophils, but also inhibit the ability of TNF-α to do so (11).

The inhibition of TNF-α-induced MIP-1α protein production by opsonized zymosan, S. cerevisiae, MSU, or CPPD suggests several possibilities. First, these agonists may inhibit secretion, but not translation of MIP-1α. This possibility was eliminated by assessing the intracellular level of MIP-1α. This was not altered regardless of the stimulation conditions (not shown). Second, MIP-1α may be degraded by these agonists, or they may be interfering with the detection of MIP-1α by ELISA. This possibility was also eliminated by conducting experiments in which neutrophils incubated with these agents were spiked with known quantities of MIP-1α, incubated for 24 h under the various conditions at 37°C, and then subjected to ELISA. The results of these experiments clearly showed that these agents neither interfered with the immunodetection of MIP-1α nor degraded the chemokine (not shown). A third possibility is that these phagocytic agonists may inhibit MIP-1α translation, fail to activate MIP-1α translation, or both. With respect to this possibility, while we cannot determine whether the agonists activate MIP-1α gene transcription without activating translation, it is possible that they inhibit translation of the MIP-1α gene, since coincubation of neutrophils with zymosan, S. cerevisiae, MSU, or CPPD and TNF-α effectively inhibits MIP-1α production by TNF-α. Moreover, both zymosan and S. cerevisiae induced accumulation of mRNA for MIP-1α, although no immunoreactive MIP-1α was detected. We are presently conducting studies to determine the mechanism by which such a translational inhibition may occur.

Acknowledgments

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References
