Inflammatory Microcrystals Differentially Regulate the Secretion of Macrophage Inflammatory Protein 1 and Interleukin 8 by Human Neutrophils: A Possible Mechanism of Neutrophil Recruitment to Sites of Inflammation in Synovitis

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Summary

Human neutrophils at inflammatory sites may be an important source of the chemotactic cytokines macrophage inflammatory protein 1α (MIP-1α; a C-C chemokine) and interleukin 8 (IL-8; a C-X-C chemokine). In this study, we show that the inflammatory microcrystals monosodium urate monohydrate (MSU) and calcium pyrophosphate dihydrate (CPPD), the major mediators of gout and pseudogout, differentially regulate the production of these two chemokines by human neutrophils. Both MSU and CPPD increased the secretion of IL-8 by neutrophils in a dose- and time-dependent manner, but had no effect on that of MIP-1α. Since inflammatory cytokines are likely to be present in the synovium during crystal-induced inflammation, we examined the interaction between TNF-α and GM-CSF and the crystals. Both TNF-α and GM-CSF stimulated IL-8 production; however, only TNF-α exerted a significant effect on MIP-1α secretion in neutrophils. IL-8 production induced by TNF-α and GM-CSF was synergistically enhanced in the presence of MSU or CPPD, whereas MIP-1α secretion induced by TNF was completely inhibited in the presence of either MSU or CPPD. Interestingly, no interaction between the crystals and the inflammatory cytokines was observed with respect to synthesis of the C-X-C chemokine MGSA in neutrophils. These results suggest that the combination of TNF-α and GM-CSF with MSU or CPPD will lead to the production of IL-8 by neutrophils and abolish the release of MIP-1α, an event that will theoretically lead to recruitment of neutrophils but not mononuclear cells. These results are in accordance with the pathological state of gout and pseudogout, where the predominant inflammatory cell is the neutrophil.

The chemokine gene superfamily is a group of small molecular weight cytokines whose synthesis is induced in various cells by inflammatory stimuli. Members of this new family of chemotactic cytokines are related by predicted primary structural similarities and by the conservation of a four-cysteine motif (1–3). There are two branches to the superfamily, classified according to the position of the first two cysteines in the conserved motif. The "C-X-C" branch, which includes PF-4, IL-8, melanocyte growth-stimulatory activity (MGSA[1] or gro), macrophage inflammatory proteins (MIP) 2α and β (also known as groβ and γ), neutrophil-activating peptide 2 (NAP-2), and ENA-78 is characterized by the separation of the first two cysteines by an intervening amino acid. In the second branch, "C-C", which contains monocyte chemotactic proteins (MCP) 1–3, RANTES, MIP-1α and β, and I-309, the first two cysteines are adjacent.

The major biological significance of the various members of the chemokine superfamily is their ability to mediate the recruitment of both overlapping and specific subsets of leukocytes as demonstrated in vitro. In general, members of the C-X-C chemokine subfamily are chemotactic for neutrophils but not for mononuclear leukocytes. In contrast, the C-C chemokines are chemotactic factors for various mononuclear cells and granulocytes other than neutrophils (1–6). These biological actions indicate that chemokines may play important roles in the development of acute and chronic inflammatory disorders. Indeed, during the last

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[1] Abbreviations used in this paper: CPPD, calcium pyrophosphate dihydrate; MGSA, melanocyte growth-stimulatory activity; MIP, macrophage inflammatory protein; MSU, monohydrate; NAP, neutrophil-activating peptide.

several years, various members of the chemokine gene superfamily have been identified in diseases such as rheumatoid arthritis, lung disease, and arteriosclerosis (1–3, 7–9).

The deposition of monosodium urate monohydrate (MSU) and calcium pyrophosphate dihydrate (CPPD) microcrystals in articular and periarticular tissues is the cause of acute or chronic inflammatory responses known as gout and pseudogout, respectively (10, 11). The clinical symptoms of these inflammatory responses are characterized by severe pain, edema, and erythema in the joint. In addition, one of the characteristics of these diseases is the massive recruitment of neutrophils to the inflammatory site. In contrast, mononuclear cells are rarely found in the inflamed joint in these arthropathies during the acute phases of these diseases. The fact that neutrophils are present in a massive number within the inflamed joint of patients suffering from gout or pseudogout suggests that these cells may play a major role in mediating the inflammatory response in these arthropathies (10, 11). Defining the mechanism(s) involved in neutrophil recruitment to the joint in gout and pseudogout is therefore of importance for the understanding of the initiating events of the acute inflammatory response.

While the principal biological activity of IL-8, that of a neutrophil chemotactic and activating factor (1, 2), suggests that it may be an important mediator of neutrophil recruitment in gout, the effect of microcrystals on chemokine production in general and of IL-8 in particular by neutrophils has not been reported. We have therefore investigated the effect of MSU and CPPD on the production of chemokines by neutrophils. In addition, since TNF-α and GM-CSF are among the most potent activators of de novo protein synthesis in neutrophils (12, 13) and are likely to be present in the synovial fluid in gout, we examined the possibility that they interact with the microinflammatory crystals to control the production of chemokines.

Materials and Methods

Reagents and Microcrystal Preparation. Human rTNF-α was a generous gift from Knoll Pharmaceuticals (Whippany, NJ). Human rGM-CSF used in this study was a generous gift from the Genetics Institute (Boston, MA). TNF-α and GM-CSF stock were stored at −80°C in PBS containing 0.01% BSA. Ficoll-Paque and Dextran T-500 were from Pharmacia (Dorval, Québec, Canada). Hyclone FCS was purchased from Professional Diagnostics (Edmonton, Alberta, Canada). Hybond N membranes, [γ-³²P]ATP and [α-³²P]dCTP were obtained from Amersham Canada (Oakville, Ontario, Canada). TNF-α and GM-CSF were free of endotoxins, as assessed by the Limulus Assay (Whittaker M.A. Bioproducts, Walkersville, MD). All the other reagents used in this study were of molecular biological grade and obtained from Sigma Chemical Co. (St. Louis, MO). MSU and CPPD microcrystals were generously provided by Dr. R. De Médicis (Unité des Maladies Rhumatismales, Centre Hospitalier de l'Université de Sherbrooke, Sherbrooke, Québec, Canada) prepared by modifications of previously described methods (14).

Isolation of Human Neutrophils. Whole blood was obtained by venipuncture, collected into tubes containing heparin, and after Dextran sedimentation, neutrophils were purified by centrifugation on Ficoll-Paque cushions (15).

Isolation of Cytoplasmic RNA and Northern Blot Analysis. After activating cells with a proper stimulus, total RNA was prepared by the RNAZol method as recommended by the supplier and Northern blots were performed as described previously (12, 13, 16). The cDNA probes used in this study were radiolabeled with [α-³²P]dCTP using the random primers DNA labeling system (GIBCO BRL, Burlington, Ontario, Canada). To confirm equal loading of RNA, the membranes were rehybridized with a synthetic oligonucleotide for 28S ribosomal RNA as described previously (16). The Northern blots shown in this report are taken from one experiment that is representative of at least four others that were performed with similar results on neutrophils from different donors. Some donor-to-donor variability with respect to the relative levels of TNF-α– and GM-CSF–induced gene expression was observed.

ELISA for IL-8, MGSA, and MIP-1α Chemokines. Human neutrophils were isolated and resuspended at 10⁷ cells per ml in 4-ml sterile tubes and were treated as described in the figure legends. The supernatants were collected and analyzed for the chemokine content. The cell pellets were washed three times in ice-cold sterile PBS and were lysed by three consecutive freeze-thaw cycles. IL-8 levels were measured using IL-8 mAb sandwich ELISA using two anti–IL-8 mAbs recognizing different noncompeting determinants as previously described (16). MIP-1α and MGSA levels were assessed using ELISA kits purchased from R & D Systems, Inc. (Minneapolis, MN).

Results

Effect of MSU and CPPD on IL-8 and MIP-1α mRNA Expression in Human Neutrophils. Human neutrophils were incubated for 3 h at 37°C with MSU, CPPD (both at 1.0 mg/ml), TNF-α (1,000 U/ml), or GM-CSF (1 nM), total RNA was prepared, and Northern blots were performed to assess chemokine mRNA expression. The concentrations of these agonists were chosen on the basis of previous studies (12, 13, 17). The filters were sequentially hybridized with cDNAs encoding different chemokines. The 3-h time point was chosen because extensive studies have indicated that it is not possible to extract RNA from neutrophils that have been incubated with crystals for longer than 3 h. Unstimulated cells contained no mRNA for any of the chemokine genes tested (RANTES, MCP-1, MCP-2, MIP-1α, MIP-1β, and I-309), with the exception of IL-8 (Fig. 1). An increase in the IL-8 mRNA level, however, was observed in response to TNF-α and GM-CSF. Neither MSU nor CPPD stimulated a detectable increase in the level of chemokine mRNA expression after 3 h of stimulation; however, MIP-1α and MIP-1β mRNA (but not mRNA for any other chemokine tested) was detectable in neutrophils stimulated with TNF-α.

Effect of MSU and CPPD on IL-8 and MIP-1α Secretion by Human Neutrophils. We next assessed the level of IL-8 and MIP-1α protein synthesis. Neutrophils were incubated with increasing concentrations of MSU and CPPD for 20 h at 37°C. Since the Northern blots showed that neutrophils contain mRNA for IL-8 and MIP-1α, the supernatants were collected and analyzed for the presence of IL-8 and MIP-1α (Fig. 2). Both MSU and CPPD stimulated the synthesis of immunoreactive IL-8 in a dose-dependent man-
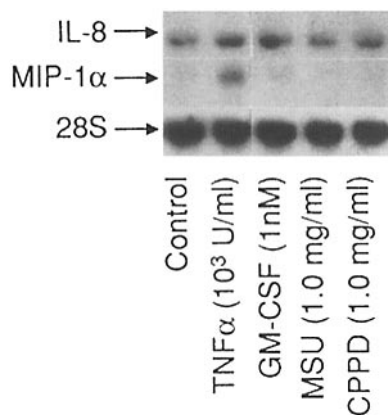
**Figure 1.** Effect of microcrystals and cytokines on IL-8 and MIP-1α gene expression. Neutrophils were incubated with diluent, TNF-α (1,000 U/ml), GM-CSF (1 nM), MSU (1.0 mg/ml), and CPPD (1.0 mg/ml) in RPMI 1640 supplemented with 1% FCS for 3 h at 37°C. Total RNA was then prepared and Northern blots were performed to detect chemokine mRNA. To confirm equally loading of RNA, the membranes were rehybridized with a synthetic oligonucleotide for 28S ribosomal RNA as described previously (16). The results shown in this figure are from one experiment that is representative of four others performed on neutrophils from different donors with similar results.

ner with a maximal effect being detected with between 0.3 and 1.0 mg/ml. In contrast, no immunoreactive MIP-1α was detected although TNF-α–stimulated MIP-1α production in these experiments (not shown).

To examine the effect of time of exposure of neutrophils to the crystals, the cells were incubated with diluent, MSU, or CPPD (both at 1.0 mg/ml) at 37°C for increasing periods of time. The cells were also treated with TNF-α (1,000 U/ml), GM-CSF (1 nM) under the same conditions. The supernatants were collected and analyzed for the presence of IL-8 and MIP-1α protein. A time-dependent release of IL-8 was observed in response to incubation with all four
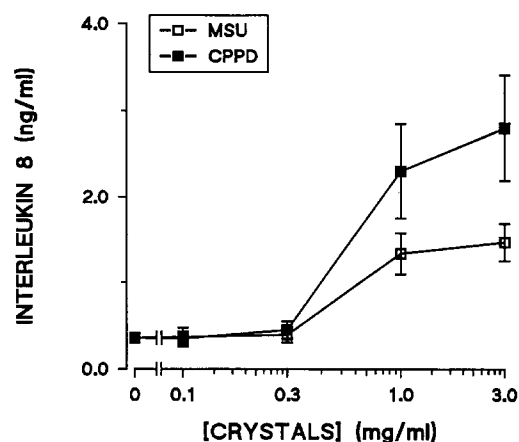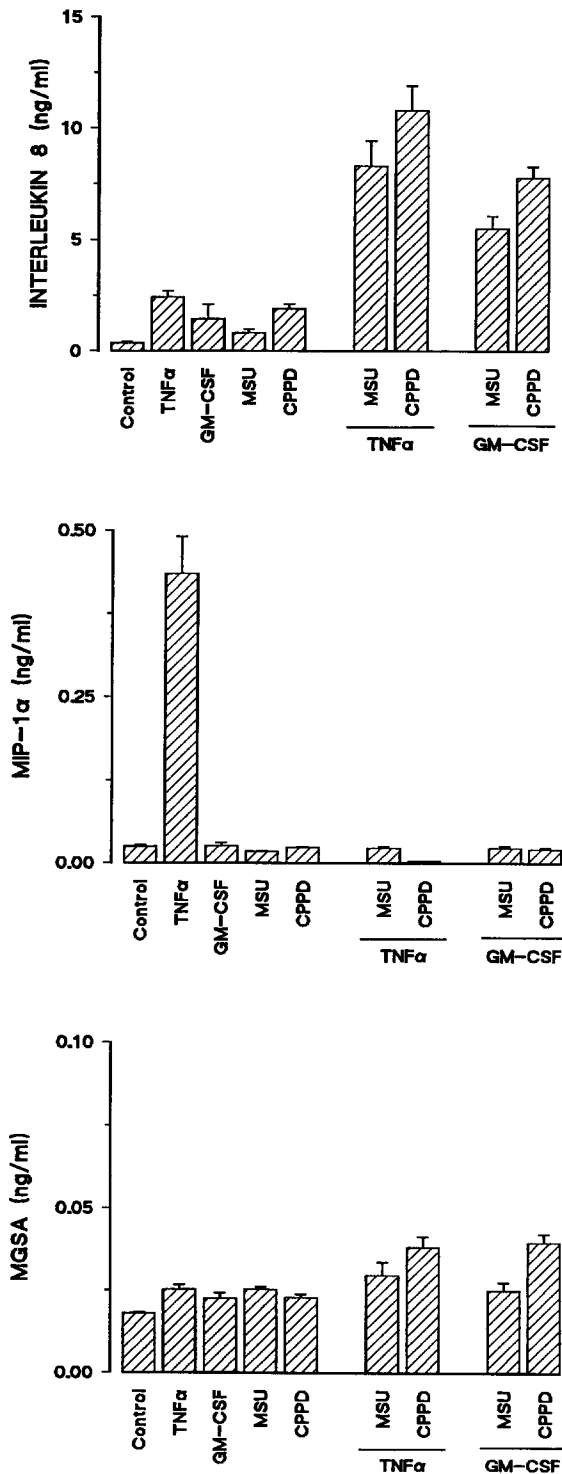

Figure 2. Effect of concentration of inflammatory microcrystals on chemokine secretion by human neutrophils. Supernatants from neutrophils incubated with either diluent, MSU (1.0 mg/ml), or CPPD (1.0 mg/ml) were collected after different periods of time and analyzed for immunoreactive IL-8 or MIP-1α protein. The results are the mean ± SEM of triplicate determinations from five separate experiments.
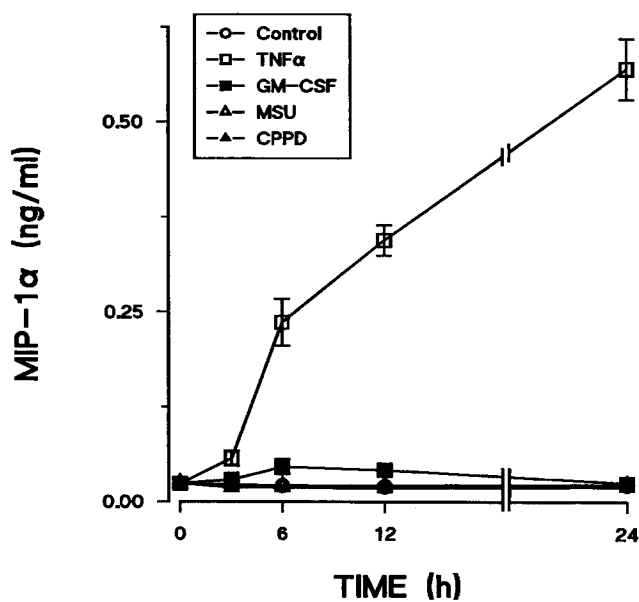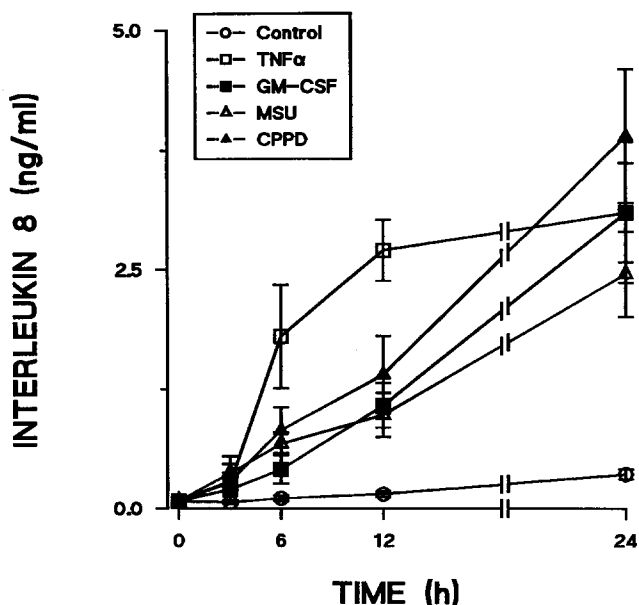
Figure 3. Effect of time of exposure to inflammatory microcrystals on chemokine secretion by human neutrophils. Supernatants from neutrophils incubated with either diluent, MSU (1.0 mg/ml), CPPD (1.0 mg/ml), TNF-α (1,000 U/ml), or GM-CSF (1 nM) were collected after different periods of time and analyzed for immunoreactive IL-8 or MIP-1α protein. The results are the mean ± SEM of triplicate determinations from six separate experiments.

agonists (Fig. 3 A). Maximal release was obtained after 24 h of incubation, the longest period of time that was assessed. In contrast, no immunodetectable MIP-1α was observed in the supernatants of neutrophils incubated with GM-CSF, MSU, or CPPD. Neutrophils incubated with TNF-α, however, showed a time-dependent secretion of MIP-1α, which continued to increase after 24 h of stimulation (Fig. 3 B).
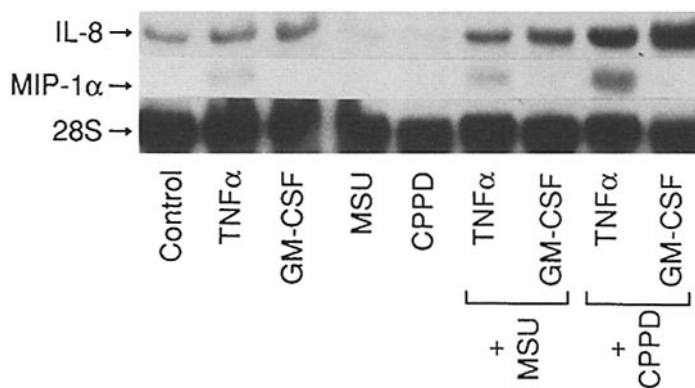
Figure 4. Effect of combination of inflammatory microcrystals and cytokines on IL-8, MIP-1α, and MGSA protein secretion by neutrophils. Neutrophils were incubated in RPMI 1640 supplemented with 1% FCS for 1 h at 37°C with TNF-α or GM-CSF and then stimulated with MSU or CPPD for up to 12 h. The same agonist concentrations were used as indicated in Figs. 1 and 2. The supernatants were collected and analyzed for the presence of IL-8, MIP-1α, or MGSA by ELISA. The results are the mean ± SEM of triplicate from five separate experiments.

Combined Effects of Microcrystals and Inflammatory Cytokines on IL-8, MGSA, and MIP-1α Secretion. Since neutrophils at sites of gouty inflammation are likely to be exposed simultaneously to crystals and cytokines, we examined the effect of such combinations on IL-8 secretion and compared it to that of MIP-1α. Neutrophils were therefore incubated at 37°C with diluent, MSU, or CPPD alone or in combination with TNF-α or GM-CSF for 12 h. The supernatants were collected and the amount of immunoreactive IL-8 and MIP-1α was determined by specific ELISA (Fig. 4). The effect of agonists alone showed the same pattern of IL-8 production shown in Fig. 3. Neutrophils were pretreated with either TNF-α or GM-CSF for 1 h at 37°C before stimulation with MSU or CPPD, and they were then incubated for 12 h at 37°C. Supernatants were collected and the amount of IL-8 protein was assessed by ELISA. A synergistic effect at the level of IL-8 production was seen in neutrophils treated with TNF-α or GM-CSF and then stimulated with microcrystals compared to the effect of the microcrystals or cytokines alone (Fig. 4). In contrast, MIP-1α production occurred only in neutrophils that were stimulated with TNF-α alone. The combination of TNF-α with either MSU or CPPD led to the complete inhibition of the production of MIP-1α protein induced by TNF (Fig. 4). To determine the generality of the effect of the combination of the agonists on C-X-C chemokine expression, we assessed the level of MGSA, a C-X-C chemokine that is closely related both structurally and functionally to IL-8, under the same conditions. Very low levels of MGSA were detected in both control and stimulated cell supernatants and, unlike that observed for IL-8, the crystals and cytokines failed to interact synergistically in the induction of MGSA secretion (Fig. 4).

Effect of Combination of Microcrystals and Inflammatory Cytokines on IL-8 and MIP-1a Gene Expression. To determine whether the inhibitory effect that was observed at the level of MIP-1α production was extended to mRNA expression, Northern blots were performed. Neutrophils were incubated under the same conditions as described for the experiments shown in Fig. 4, except that the period of incubation was 3 h instead of 24 h. Representative Northern blots are shown in (Fig. 5). Stimulation of the cells with TNF-α or GM-CSF together with either MSU or CPPD resulted in an additive or synergistic increase in the level of IL-8 mRNA. In contrast to that observed at the level of MIP-1α secretion, however, the effect of combining TNF-α with either MSU or CPPD did not inhibit the expression of MIP-1α mRNA induced by TNF-α, but rather enhanced it (Fig. 5).

Discussion

The results of this study show for the first time that the inflammatory microcrystals MSU and CPPD stimulate the production of IL-8 in human neutrophils. In addition, evidence was obtained demonstrating that under the same conditions, production of C-C chemokines, chemotactic factors for mononuclear cells, does not occur. Because IL-8

Figure 5. Effect of combinantion of inflammatory microcrystals and cytokines on IL-8 and MIP-1α mRNA expression in neutrophils. Neutrophils were pretreated with TNF-α or GM-CSF for 1 h and then stimulated for 3 h at 37°C with either MSU or CPPD (both at 1.0 mg/ml). Total RNA was prepared and Northern blots were performed to determine the expression of IL-8 and MIP-1α mRNA. The results shown in this figure are from one experiment that is representative of three others performed on neutrophils from different donors with similar results.

has been shown to mediate exclusively the recruitment of neutrophils in vivo, these results have important implications in gout and pseudogout, inflammatory arthropathies in which the major infiltrating cell is the neutrophil.

Inflammatory microcrystal deposition on articular or periarticular joints is believed to be the major cause of arthropathies such as gout and pseudogout, diseases characterized by synovial inflammation (10, 11). In addition, several lines of evidence support the concept that the neutrophil is the major effector cell in acute gouty inflammation: (a) Neutrophils, which accumulate in massive numbers in both synovial fluid and synovial membrane in gout, are the predominant inflammatory cell in the joint. (b) Experimental microcrystal-induced synovitis is greatly diminished in neutrophil-depleted animals (18, 19). (c) Inflammatory microcrystals are potent activators of signal transduction pathways and effector function in neutrophils. For instance, incubation of neutrophils with MSU or CPPD leads to a rapid intracellular calcium mobilization (20), activation of phosphatidylcholine-specific phospholipase D (21), formation of inositol 1,4,5 triphosphate (22), and an increase in protein tyrosine phosphorylation (23). Neutrophils stimulated with MSU or CPPD also release oxygen–derived free radicals (24), and cytokines such as IL-1 and IL-1 receptor agonist (17). These previous observations suggest that neutrophils that are recruited to the synovial space during a gouty attack will be activated when exposed to inflammatory microcrystals; however, the results of the present study imply that under the same conditions, neutrophils will also contribute significantly to their own recruitment because of their ability to release high levels (nanogram quantities) of IL-8. In addition, while production of IL-8 by neutrophils exposed to inflammatory microcrystals was observed and was synergistically enhanced when neutrophils were exposed to inflammatory microcrystals in the presence of TNF-α or GM-CSF, production of the closely-related C-X-C chemokine MGSA was not affected. In contrast to IL-8, MGSA was only detected in low picogram quantities. This was the case regardless of whether neutrophils were incubated with the crystals alone or in combination with either TNF-α or GM-CSF.

IL-8 is a prominent member of the C-X-C branch of chemokine gene superfamily. The biological importance of

C-X-C chemokines stems from the fact that they are potent chemotactic and activating factors for neutrophils. For instance, IL-8 stimulates neutrophil chemotaxis both in vitro and in vivo (1–3) and it activates a variety of neutrophil functions including calcium mobilization, the respiratory burst, degranulation, adhesion, and leukotriene synthesis (1–3). In contrast, the biological actions of the members of the C-C subfamily, including MIP-1α, are directed towards mononuclear cells and granulocytes other than neutrophils. While early reports showed that MIP-1 exerts a stimulatory effect on human neutrophils, those studies were performed using a mixture of purified (not recombinant) muMIP-1α and muMIP-1β (25). We have recently shown that in contrast to previous reports of the effect of muMIP-1, neither recombinant huMIP-1α nor huMIP-1β possess significant stimulatory activity towards human neutrophils in terms of chemotaxis, degranulation, activation of the Na+/H+ antiport, or actin polymerization (18). They do, however, stimulate intracellular calcium mobilization and right-angle light scatter via a pertussis toxin-sensitive G protein–dependent mechanism (18). Since the latter two functions are among the most sensitive measures of neutrophil activation (26), we concluded that neutrophils were not the major targets for the biological actions of huMIP-1α and β, a conclusion that supports the paradigm that the major biological targets of the members of the C-C branch of the chemokine superfamily are mononuclear cells and not neutrophils.

The fact that the inflammatory cells present within an inflamed joint in crystal-induced synovitis are predominantly neutrophils and not mononuclear cells, a state that is histologically different to that of patients suffering from rheumatoid arthritis, a chronic inflammatory arthropathy, suggests that the influx of mononuclear cells during acute gouty inflammation may be preferentially inhibited or prevented. The results of the present study clearly demonstrate that neutrophils exposed to inflammatory microcrystals produce IL-8, thereby providing an autocrine mechansim for amplification of neutrophil recruitment to the gouty joint. By showing a concommitant inhibition of MIP-1α production, however, they also provide for the first time a mechanism that may inhibit the recruitment of mononuclear cells. This observation is important when considering that

MIP-1α and β may be the major, if not the only, C-C chemokines produced by activated neutrophils (reference 27 and this study). Indeed, in this study, Northern blot analysis failed to detect mRNA for any other known C-C chemokine tested, including MCP-1, MCP-2, MCP-3, RANTES, or I-309 in unstimulated cells or in cells incubated with TNF-α, GM-CSF, MSU, or CPPD, either alone or in combination. These results therefore raise the possibility that the failure of inflammatory microscrystals to directly induce MIP-1α (and probably MIP-1β) production, as well as their ability to inhibit the production of MIP-1α by neutrophils in response to TNF-α, may prevent the generation of a chemotactic signal by neutrophils that could potentially attract a wide range of mononuclear cells, including monocytes and lymphocyte subsets (4–6) to the synovial environment.

While the mechanism by which MSU and CPPD inhibit MIP-1α production is unclear, it appears from our studies that the inhibitory effect is mediated at the posttranscriptional or translational level. This conclusion is supported by our experiments showing that while the inflammatory microcrystals inhibited the production of immunodetectable MIP-1α induced by TNF-α, mRNA for MIP-1α was either unaffected or enhanced. This suggests, although does not unequivocally prove, that MIP-1α gene transcription is not being inhibited. We have also determined that the crystals do not enhance the degradation of MIP-1α protein or interfere with the immunodetection of MIP-1α by ELISA (not shown). Taken together, these data suggest that the inhibitory effect is mediated at the translational level. Further studies will be required to determine the specific mechanism of inhibition.

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