Characterization of an Activation Factor Released from Human Neutrophils After Stimulation by Triclinic Monosodium Urate Crystals

PHILIPPE DESAULNIERS, SÉBASTIEN MAROIS, GUILLAUME PARÉ, OANA POPA-NITA, CAROLINE GILBERT, and PAUL H. NACCACHE

ABSTRACT. ***Objective.*** To determine the presence and characterize the activity of a soluble activation factor rapidly released by human neutrophils after stimulation with monosodium urate (MSU) crystals.

Methods. Supernatants from human neutrophils stimulated by MSU crystals for 5 to 60 min were tested for their ability to stimulate a chemotactic response, induce a mobilization of calcium, and increase the tyrosine phosphorylation levels in naive neutrophils.

Results. Supernatant from neutrophils stimulated ≤ 15 min by MSU crystals was chemotactic for neutrophils, induced a mobilization of calcium, and increased the levels of tyrosine phosphorylation in fresh neutrophils. Generation of activity in the supernatant was independent of protein synthesis and was eliminated after digestion with trypsin. Leukotriene B_4 (LTB_4), platelet-activating factor (PAF), and formyl peptide receptor antagonists as well as neutralizing anti-interleukin 8 (IL-8) antibodies did not inhibit the chemotactic activity in the supernatant, although pertussis toxin did inhibit the mobilization of calcium observed in response to the supernatant. Stimulation of neutrophils with formyl-methionine-leucine-phenylalanine, IL-8, and LTB_4 inhibited subsequent mobilization of calcium by the supernatant.

Conclusion. There is rapid liberation of a potent activation signal from neutrophils after interaction with MSU crystals. This activation factor can further stimulate surrounding neutrophils and contribute to amplification of the inflammatory response induced by MSU crystals. (J Rheumatol 2006;33:928–38)

Key Indexing Terms:
NEUTROPHILS HUMAN GOUT CHEMOTAXIS
MONOSODIUM URATE CRYSTALS TYROSINE PHOSPHORYLATION

The polymorphonuclear neutrophil is the first leukocyte to accumulate in large numbers at the inflammatory site. Neutrophils can be recruited in response to multiple stimuli, endogenous as well as exogenous, including complement components (C5a), bacterial peptides [formyl-methionine-leucine-phenylalanine (fMLF)], chemokines [interleukin 8 (IL-8), growth-related oncogene-α (Gro-α), nerve action potential-2 (NAP-2)], lipid mediators [leukotriene B_4 (LTB_4), platelet-activating factor (PAF)], and members of the S100 family, also known as myeloid related proteins (MRP)[1-3]. These signals orient the neutrophil, increase its adhesion, and induce the expression of receptors and secretion of molecules to promote inflammation and/or postpone apoptosis[4-7].

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The neutrophil is by far the most abundant leukocyte recruited to the site of inflammation in gouty arthritis. Interaction of monosodium urate (MSU) microcrystals with cells in the joints leads to the production of inflammatory mediators such as IL-8[8], LTB_4 and LTC_4[9,10], PAF[11], Gro-α[12], and C5a[13], one or more of which can play a role in amplification of the recruitment of neutrophils. The accumulation and activation of neutrophils are associated with tissue damage caused by the release of neutrophil granules, reactive oxygen species, and proteolytic enzymes. Ingestion of the crystals by neutrophils also leads to the release of inflammatory mediators[14-16] that perpetuate the destructive cycle. The interaction of MSU crystals with neutrophils is thought to be mediated in part by the Fcγ receptor CD16b (with the participation of the CD11b/CD18 complex[17]) and leads to colchicine-dependent robust increases in tyrosine phosphorylation[18,19], mediated in part by the tyrosine kinase Syk[17], in the cytoplasmic concentration of free calcium[16], and in the activities of PI3 kinase[20] and phospholipases D[21] and A_2[22].

Despite the vigorous and rapid responses observed in suspensions of neutrophils exposed to MSU crystals, previous investigators reported that as little as 15% of neutrophils contained MSU crystals after 8 minutes, a fraction that increased to only 41% after 30 minutes[23], while other authors noted

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50% of the cells ingesting MSU crystals after incubations of up to 60 minutes[24]. This led us to examine whether the responses summarized above, which are mostly detected within seconds or at most minutes of the addition of MSU crystals, were due to the direct interaction of MSU crystals with neutrophils or instead to factors secreted by neutrophils in contact with the crystals.

We produced supernatants from MSU-stimulated cells (called SMC) and examined their effects on naive neutrophils. We noted significant activation of neutrophils by SMC that included increases in tyrosine phosphorylation, a mobilization of calcium, and induction of a chemotactic response. The basic characteristics of the activity of SMC differentiate it from lipid mediators, IL-8, and formylated peptides as well as from the previously reported crystal-induced chemotactic factor (CCF)[25-27]. Our data indicate that even very rapid responses of human neutrophils to MSU crystals may result from the additive effects of direct contact of MSU crystals with the cells and from the activity of SMC.

MATERIALS AND METHODS

Reagents. Ficoll-Paque was obtained from Wisent Canadian Laboratories (St-Bruno, Québec, Canada). The enhanced chemiluminescence (Renaissance) reagents used for immunoblotting were from DuPont Pharmaceuticals (Mississauga, ON, Canada). PP2 was purchased from Calbiochem (San Diego, CA, USA) and Biomol (Plymouth Meeting, PA, USA). Pertussis toxin was purchased from List Biologicals (Campbell, CA, USA). Dextran T-500 was purchased from Pharmacia (Baie d'Urfé, Québec, Canada). p-Nitrophenylphosphate, aprotinin, and leupeptin were purchased from ICN Pharmaceuticals (Costa Mesa, CA, USA). Cycloheximide and trypsin were obtained from Sigma-Aldrich (Oakville, ON, Canada). LTB_4 and PAF were generous gifts of Dr. Pierre Borgeat (Laval University, Québec, Canada). BN 50730 was a generous gift from the Institut Henri Beaufour (Paris, France). Cyclosporin H originated from Novartis Pharma AG (Basel, Switzerland). Triclinic MSU crystals were kindly provided by Drs. R. de Médicis and A. Lussier (University of Sherbrooke, Sherbrooke, Québec, Canada) and prepared as described[15]. The crystals used in this study were characterized by X-ray diffraction (Geigerflex D/max, Rigaku, Wakefield, MA, USA) and examined under phase and polarization microscopy and by scanning electron microscopy. Several distinct lots of crystals (sizes between 10 and 20 μM, specific areas between 0.7 and 2.4 m^2/g) were used with identical results (data not shown). CP 105.696 was provided by Pfizer Central Research (Groton, CT, USA).

Antibodies. Peroxidase-labeled anti-mouse (no. 115-095-072) antibodies were obtained from Jackson ImmunoResearch (West Grove, PA, USA). The anti-phosphotyrosine antibodies (no. UBI-05-321, clone 4G10) were purchased from Upstate Biotechnology (Lake Placid, NY, USA). Phosphospecific ($pTpY^{185/187}$) anti-phosphoERK1/2 (no. 44-680) antibodies were obtained from Biosource International (Camarillo, CA, USA). Anti-human IL-8 antibody (no. 500-M08) was purchased from Peprotech Canada Inc. (Ottawa, ON, Canada).

Neutrophil purification. Blood was obtained from the peripheral vein of healthy adults as described[28]. Neutrophils were obtained by means of 2% Dextran sedimentation followed by standard Ficoll gradient techniques. Contaminating erythrocytes were removed by hypotonic lysis, and purified granulocytes (> 95% neutrophils, < 5% eosinophils, < 0.1% monocytes) were resuspended in Hanks' balanced salt solution (HBSS) containing 1.6 mM calcium and no magnesium (pH 7.4) without serum. The isolation procedure was carried out under sterile conditions.

Production of SMC. Neutrophils (4×10^7 cells/ml in HBSS, 700 μl) were incubated in microcentrifuge tubes with MSU crystals (3 mg/ml) for 5–60 min at 37°C with agitation and then pelleted (6000 g for 15 s) and the supernatants were collected and filtered through a 0.22 μm filter. Special care was taken while collecting the supernatants to avoid contamination with neutrophils or MSU crystals. Control supernatants were also similarly prepared from unstimulated cells.

Tyrosine phosphorylation. Neutrophil suspensions (4×10^7 cells/ml) were either incubated at 37°C with MSU crystals (3 mg/ml) or resuspended in supernatants from MSU-stimulated cells (SMC). The reactions were stopped by the addition of cell aliquots to an equal volume (100 μl) of boiling 2× Laemmli sample buffer [1× is 62.5 mM Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate (SDS), 5% ß-mercaptoethanol, 8.5% glycerol, 2.5 mM orthovanadate, 10 mM paranitrophenylphosphate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.025% bromophenol blue] and boiled for 7 min. Samples were then subjected to 7.5–20% SDS-polyacrylamide gel electrophoresis and transferred to Immobilon PVDF membranes (Millipore, Bedford, MA, USA). Immunoblotting was performed using the 4G10 antiphosphotyrosine antibody at a final dilution of 1/4000 and revealed by the Renaissance Plus detection system as described[29].

Mobilization of intracellular calcium. Cells (10^7 cells/ml) were incubated 30 min at 37°C with 1 μM fura-2/AM (Molecular Probes, Eugene, OR, USA). The neutrophils were washed once in HBSS to remove the extracellular probe, resuspended at 5×10^6 cells/ml, and transferred to the thermoregulated (37°C) cuvette compartment of a spectrofluorimeter (SLM 8000; Aminco, Urbana, IL, USA). In experiments where activity of SMC was tested, cells were resuspended after loading with Fura-2AM in 1 ml of HBSS and then 1 ml of SMC was added. The fluorescence was monitored at excitation wavelength 340 nm and emission wavelength 510 nm. The internal calcium concentrations were calculated as described[30].

Measurement of neutrophil migration. Chemotaxis was measured as described[31]. Briefly, neutrophils were resuspended in RPMI-1640 and 10% fetal bovine serum (FBS) at 10^7 cells/ml and were preincubated with 5 μg/ml calcein-AM (Molecular Probes) at 37°C for 30 min in the dark with constant agitation. Cells were washed twice and resuspended in RPMI/FBS at 5×10^6 cells/ml at 37°C. Neutrophil migration was monitored using a 96-well chemoTX disposable chemotaxis system (NeuroProbe, Gaithersburg, MD, USA). The fluorescence of cells in the filters was measured with a microplate fluorescence reader (FL600; Bio-Tek Instruments, Winooski, VT, USA; excitation wavelength 485 nm, emission wavelength 530 nm). The fluorescence from known numbers of neutrophils was obtained by placing them into the bottom chamber. The results are expressed as the number of cells that penetrated the filters.

Microscopy. The neutrophils (2×10^7 cells/ml) were incubated with MSU crystals (1.5 mg/ml) for 30 min at 37°C before being observed with a polarized light microscope.

RESULTS

The interactions between neutrophils and MSU crystals were first monitored visually by optical microscopy. To this end, neutrophils were exposed to 1.5 mg/ml MSU crystals for 30 min. Aliquots of the cell suspensions were then deposited on microscope slides and examined. As illustrated in Figure 1, only a small percentage of the cells (indicated by arrows) were observed to interact directly with the crystals, despite the presence of multiple free-floating crystals and the relatively long incubation period. Neutrophils that have ingested MSU crystals can be identified by their stretched, elongated shape and the presence of MSU crystals within their membrane. It should be pointed out that the concentration of MSU crystals used in these experiments has been shown[16,18] to elicit near-

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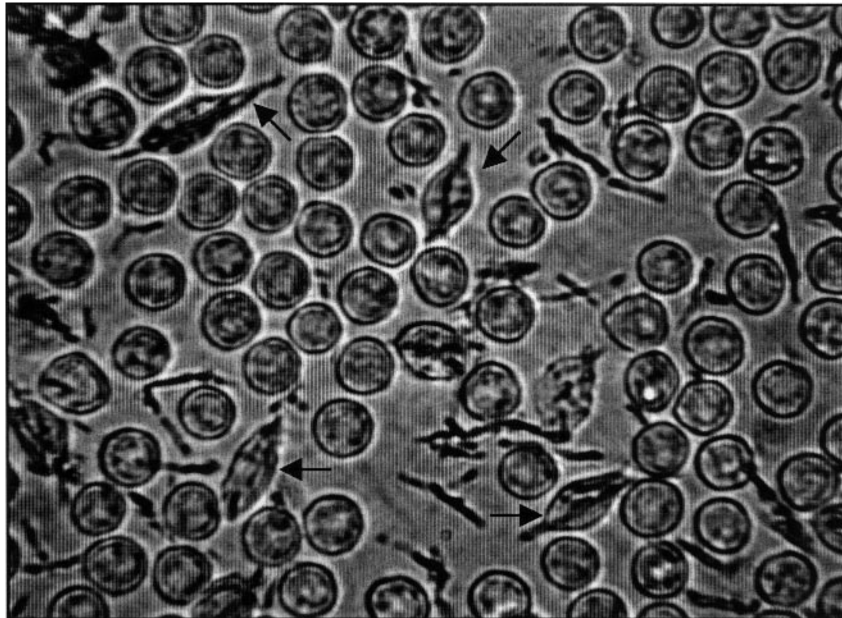

Figure 1. Visualization of neutrophil ingestion of MSU crystals. Cells (1.5×10^7 cells/ml) were stimulated with MSU crystals (1.5 mg/ml) at 37°C for 30 min. Cells were visualized under polarized light microscopy. Arrows indicate cells that have ingested MSU crystals.

maximal responses in calcium mobilization and tyrosine phosphorylation assays. These observations suggest that neutrophils exposed to MSU crystals produce activation mediators that stimulate surrounding cells.

To verify this hypothesis, we performed a chemotaxis assay using supernatants from neutrophils stimulated with MSU crystals (SMC). Neutrophils were incubated with MSU crystals for 15 to 60 min before being pelleted. The SMC were collected, filtered to eliminate free-floating cells and crystals, and then placed in the lower chamber of a ChemoTX plate for the chemotaxis assay carried out as described in Materials and Methods. There was significant chemotactic activity in response to SMC derived from incubation of neutrophils with MSU crystals for 15 min when compared to control supernatants obtained from unstimulated cells (Figure 2A). There was a slight increase of chemotactic potency in the SMC derived from incubation of neutrophils with MSU crystals for 30 to 60 min compared to SMC obtained from incubation of neutrophils with MSU crystals for 15 min. Diluting the SMC correlated with a reduced chemotactic activity (data not shown).

Calcium mobilization is involved in many signaling pathways and in the performance of multiple cellular functions. As such it is a hallmark of cell activation. Resting neutrophils loaded with Fura-2AM were stimulated with SMC obtained from incubation of neutrophils with MSU crystals for 15 to 60 min, and the concentrations of free intracellular calcium were then monitored as described in Materials and Methods. As shown in Figure 2B, SMC induced a rapid and transient mobilization of calcium in neutrophils. Generation of the calcium-mobilizing activity of SMC required as little as 15 min of incubation with MSU crystals and was not significantly increased by further incubation up to 60 min.

Preincubating cells with cycloheximide (20 μg/ml) for 30 min to block *de novo* protein synthesis before stimulating them with MSU crystals inhibited by only 20% the chemotactic response to supernatants stimulated with MSU crystals for 15 min (Figure 3A). The inhibitory effect of cycloheximide increased to 30% and 40% with supernatants from cells stimulated with MSU crystals for 30 to 60 min, respectively. It should be noted that the cycloheximide-resistant fraction of the chemotactic activity of the SMC remained constant over this time period.

We further characterized the activity of the SMC by examining whether it had the ability to affect the tyrosine phosphorylation pattern in human neutrophils. An increase in tyrosine phosphorylation in human neutrophils has been observed in response to many agonists and is closely associated with their activation status[18,32-35]. Supernatants from neutrophils stimulated by MSU crystals for as little as 5 min induced a rapid increase (within 30 s) in the pattern of tyrosine phosphorylation, with a prominent increase of the tyrosine phosphorylation of a 110–120 kDa band and minor increases in the 60–90 kDa region (Figure 3B). SMC derived from cycloheximide-treated cells was essentially as potent as SMC derived from untreated cells in its ability to stimulate a tyrosine phosphorylation response (Figure 3B). SMC also had the ability to stimulate the phosphorylation of Erk1/2 (Figure 3C).

The sensitivity of SMC to trypsin digestion was then tested. Since neutrophils contain antitrypsin activities in their granules and MSU crystals induce a significant degranulation response in neutrophils (data not shown), we boiled the SMC

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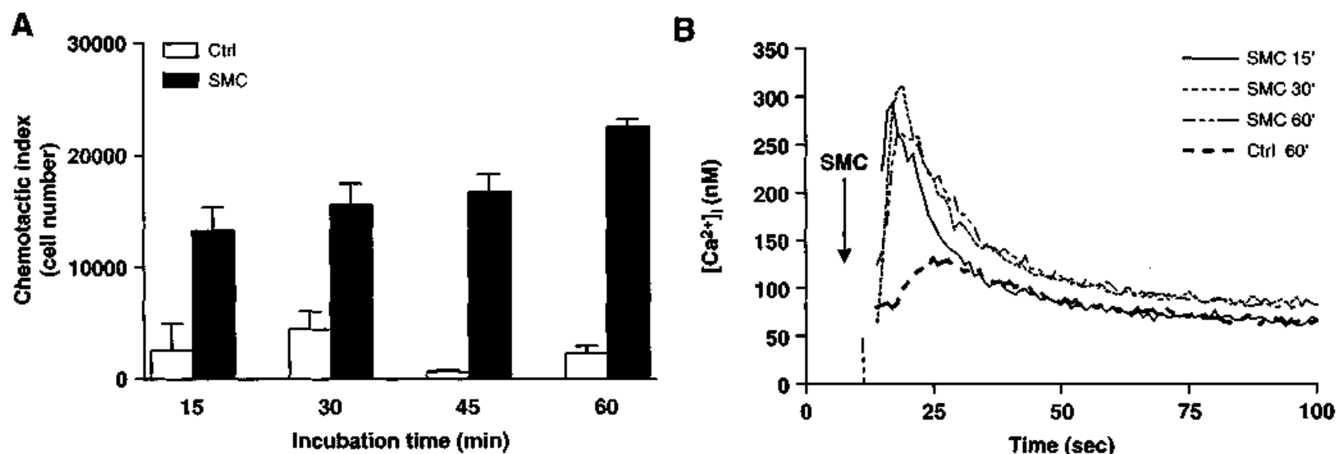

Figure 2. A. Chemotaxis and calcium mobilization induced by SMC from different incubation periods. Neutrophil migration was monitored with a ChemoTX disposable chemotaxis system. Polycarbonate filters were positioned on the plate, and neutrophils (30 μl, 60,000 cells/well) were placed on the filter and allowed to migrate in response to SMC from 15 to 60 min. Results are expressed as number of cells that penetrated the filters (n = 3), as described in Materials and Methods. B. Neutrophils (10^7 cells/ml) were loaded with 1 μM Fura-2/AM and stimulated at 5×10^6 cells/ml with 1 ml of SMC from 15–60 min incubation with MSU crystals. Data shown are representative of 3 independent experiments.

to inactivate the endogenous trypsin inhibitors, then incubated the SMC with or without trypsin (250 μg/ml) for 1 h at 37°C, before boiling the SMC again to denature the exogenous trypsin. The chemotactic activity of the SMC thus treated was then tested. The SMC treated with trypsin showed a drastically reduced chemotactic activity (data not shown). IL-8 chemotactic activity was also abrogated by trypsin digestion (data not shown).

We then submitted SMC to Centricon centrifugal filter separation using various cutoff sizes to get a rough indication of the molecular weight of the neutrophil-active factor(s). After centrifugal separation, we monitored the chemotactic activity of the flow-through and the excluded fractions. We found that the fraction above 30 kDa retained as much chemotactic activity as the starting material, while less than half the activity remained above 50 kDa and none above 100 kDa (data not shown).

Src kinases are known to be involved in phagocytosis as well as in chemotaxis[36-43]. The neutrophils incubated with MSU crystals in the presence of the potent Src kinase inhibitor PP2 retained a round shape and showed a marked reduction of MSU crystal ingestion (data not shown). Preincubating neutrophils with PP2 essentially abrogated the ability of SMC derived from these cells to induce a mobilization of calcium activity in naive neutrophils (Figure 4). It should be noted that the calcium-mobilizing activity of the SMC itself was insensitive to inhibition by PP2 (data not shown). Generation of neutrophil-stimulating activity in the SMC thus appears to be dependent on Src kinases.

The neutrophil secretes many chemotactic agents, including LTB_4, PAF, and IL-8. We next examined the potential contribution of the lipid mediators LTB_4 and PAF to the activity of the SMC. We used the LTB_4 and PAF receptor antagonists CP 105.696[44,45] and BN 50730[46], respectively. Preincubation of neutrophils with either of these 2 compounds (data not shown), or with both (Figure 5A) had no effect on the tyrosine phosphorylation response to the SMC. The activity of the receptor antagonists was verified by testing their effects on the responses to their respective lipid mediators (Figure 5B). We confirmed the lack of involvement of these 2 agonists in the activity of the SMC by observing that pyrrophenone, a cPLA2 inhibitor[47,48], had no effect on the tyrosine phosphorylation response induced by SMC and only marginally affected its chemotactic activity (data not shown). A blocking anti-IL-8 antibody was used next to determine the role of IL-8 in the activity of SMC. No significant inhibition of the chemotactic response to SMC was observed in the presence of anti-IL-8 antibodies, while the response to IL-8 was inhibited by this antibody (data not shown). The potential involvement of mitochondrial-derived formylated peptide or the granule protein cathepsin G, which has been identified as a novel chemotactic agonist that interacts with the formyl peptide receptor[49], was assessed by testing the inhibitory activity of cyclosporin H, a formyl peptide receptor-specific inhibitor[50]. We incubated neutrophils with cyclosporin H and found no inhibition of the chemotactic and mobilization of calcium responses to SMC, although the responses to fMLF were significantly decreased (Figures 5C, 5D, 5E, 5F).

Many chemotactic agents have receptors coupled to G-proteins[51]. We investigated the potential role of G-proteins in the response of human neutrophils to SMC by using pertussis toxin. The cells were incubated with 1 μg/ml pertussis toxin for 1 h, then their ability to respond to SMC, fMLF, and CD32a ligation was tested. Pertussis toxin drastically reduced the calcium responses to SMC as well as to fMLF, while it did not inhibit the calcium response to CD32a cross-linking (data not shown). These data indicate that pertussis toxin-sensitive G-proteins are involved in the mediation of responses to SMC.

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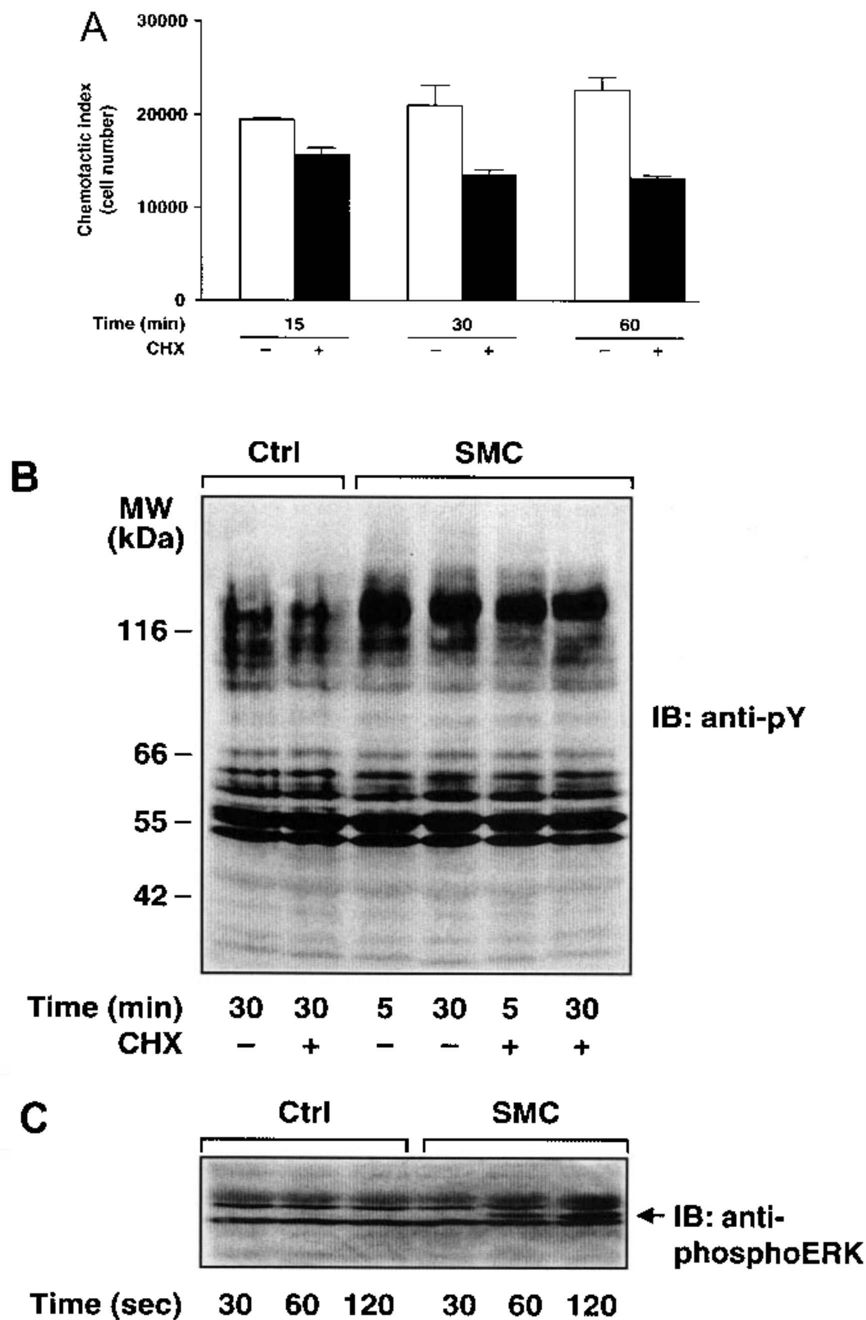

Figure 3. Production of SMC is independent of *de novo* protein synthesis. A. Cells (4×10^7 cells/ml) were preincubated with cycloheximide (CHX; 20 μg/ml) for 30 min before being stimulated with MSU crystals (3 mg/ml) for 15 to 60 min (as indicated). The SMC obtained were used in a chemotaxis assay as described in Materials and Methods (n = 3). B. Cells (4×10^7 cells/ml) were preincubated with CHX (20 μg/ml) for 30 min and stimulated with MSU crystals (3 mg/ml) for 5 to 30 min. The SMC obtained were used to stimulate naive neutrophils. Reactions were stopped after 30 s and samples were subjected to Western blot analysis (IB) with anti-phosphotyrosine (anti-pY) antibody. Data shown are representative of 3 independent experiments. C. Cells (4×10^7 cells/ml) were stimulated with MSU crystals (3 mg/ml) for 15 min. SMC obtained were used to stimulate naive cells. Reactions were stopped after 30, 60, or 120 s by transfer of a cellular aliquot to boiling sample buffer 2× and samples were subjected to Western blot analysis with anti-phosphoERK antibody. Control cells were stimulated with supernatant from unstimulated cells. Data shown are representative of 3 independent experiments.

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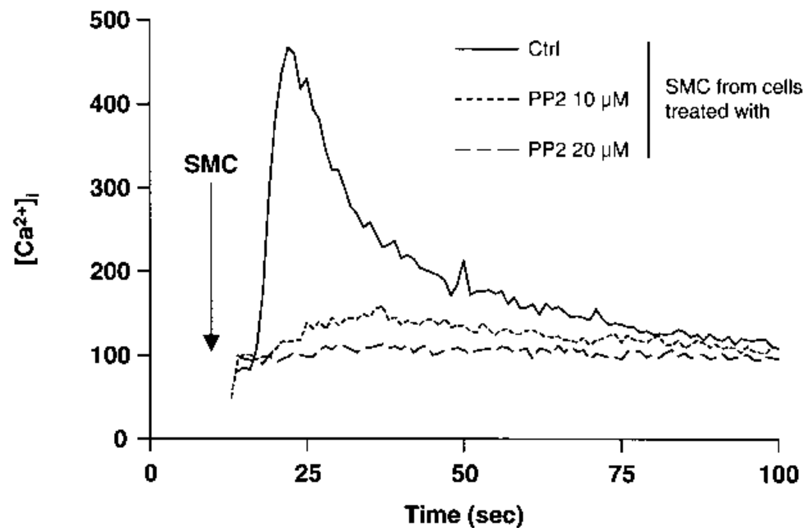

Figure 4. Calcium mobilization induced by SMC derived from PP2-treated neutrophils. Cells (10^7 cells/ml) were loaded with 1 μM Fura-2/AM as described in Materials and Methods and stimulated at 5×10^6 cells/ml with 1 ml of SMC derived from PP2 (10 and 20 μM) treated neutrophils stimulated with MSU crystals (3 mg/ml). Data shown are representative of 3 independent experiments.

Cross-desensitization experiments provided evidence that the responses of human neutrophils to SMC shared signaling pathways with those utilized by chemotactic factors. Neutrophils were first stimulated with IL-8 (10^{-7} M), LTB_4 (10^{-7} M), or fMLF (10^{-7} M) and 2 minutes later, with SMC. As can be seen in Figure 6, prior exposure to the chemotactic factors essentially abrogated the mobilization of calcium induced by SMC.

DISCUSSION

Although MSU crystals, the etiological agent of gout, are among the most potent proinflammatory stimuli, a complete account of the mechanisms underlying their phlogistic activity remains elusive. While direct effects of MSU crystals on, among others, neutrophils, monocytes/macrophages, fibroblasts, osteoblasts[52,53], and endothelial cells[54] have been described, significant gaps remain in our understanding of the inflammatory effects of MSU crystals. Our findings indicate that several of the previously reported effects of MSU crystals on human neutrophils may result, at least in part, from the indirect effects of a factor rapidly released from neutrophils exposed to MSU crystals that had previously escaped identification.

This investigation was prompted by our observation that only a small percentage of neutrophils ingested or adhered to MSU crystals within the timeframe in which most early responses to the crystals have been monitored, an observation in accord with the reported prevalence and kinetics of internalization of MSU crystals[23,24]. After stimulating neutrophils, we found that within a short time (5–15 min) a neutrophil-activating factor was clearly detectable in the supernatant of the cells. This activation factor induced an increase in tyrosine phosphorylation, stimulated the mobilization of calcium, and possessed chemotactic activity. It remains unknown whether the activities detected in the supernatants of MSU crystal-stimulated cells are due to one or more factors. Our observations favor the hypothesis of rapid (< 15 min) release of a prestored neutrophil-activating factor, followed in time (30–60 min) by that of a protein synthesis-dependent additional factor. The cycloheximide sensitivity of the increases in chemotactic activity of the SMC collected at 30 and 60 minutes, compared to SMC collected after 5–15 minutes of interaction between the cells and MSU crystals, supports this interpretation, although there was no corresponding increase in the ability of the 30–60-minute SMC to induce a mobilization of calcium or to stimulate a tyrosine phosphorylation response. Generation of SMC activity is dependent on active metabolic processes, as the Src kinase inhibitor PP2 inhibited the appearance of calcium-mobilizing activity in the SMC. On the other hand, its generation is insensitive to inhibition by colchicine (data not shown), although tyrosine phosphorylation induced by MSU crystals is inhibited by colchicine[19]. It is unclear whether the production of activity in SMC is a result of phagocytosis of MSU crystals or simply adherence to MSU crystals, although cytochalasin B (data not shown) and colchicine did not inhibit the production of SMC, which might indicate that adherence of MSU is sufficient.

Although presently unidentified, the activity detected in the supernatants of neutrophils exposed to MSU crystals shares several characteristics with classic chemotactic factors, including the pertussis toxin sensitivity of the responses to the SMC. These data correlate with indications that MSU crystals activate neutrophils through pertussis toxin-sensitive as well as insensitive pathways[55]. Calcium desensitization assays have revealed similarities and differences between the differ-

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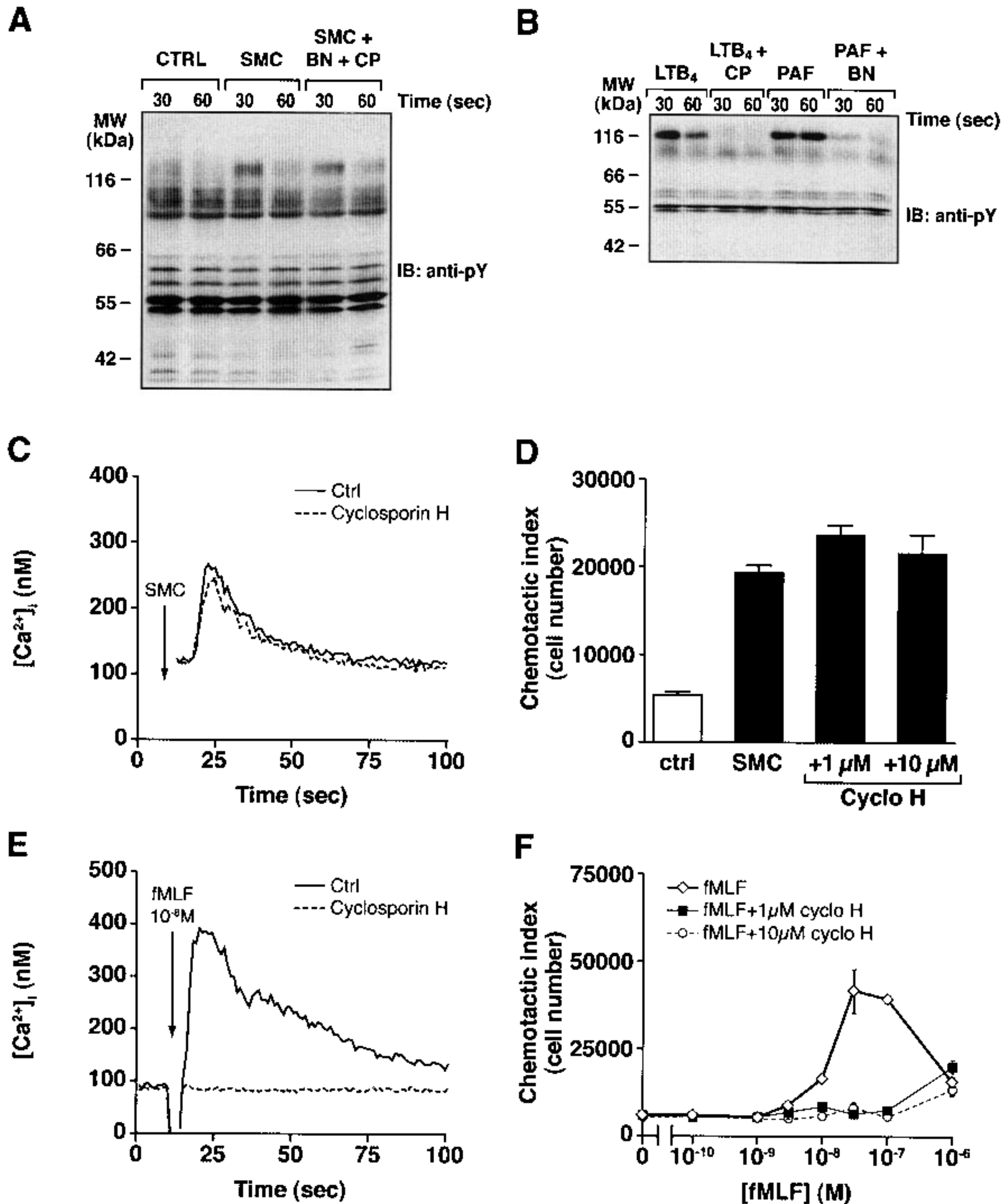

Figure 5. Effect of antagonists for LTB_4 (CP105.696) and PAF (BN 50730) receptors on the tyrosine phosphorylation induced by SMC: Cells were preincubated with CP (10^{-6} M) and BN (10^{-6} M) then resuspended in SMC (A), or stimulated with LTB_4 (10^{-7} M) or PAF (10^{-7} M) (B), as described in Materials and Methods. Samples were analyzed by Western blot (IB) with anti-phosphotyrosine antibody (anti-pY). Data shown are representative of 3 independent experiments. Effect of cyclosporin H (cyclo H) on chemotaxis and calcium mobilization induced by SMC: Cells were preincubated with cyclosporin H (1–10 μM) for 5 min, and monitored for mobilization of calcium induced in response to SMC (C) or fMLF (10^{-8} M) (E), or the chemotactic responses to SMC (D) and fMLF (F). Cyclosporin H was used at 10 μM in mobilization of calcium assays. HBSS was used as control in the chemotactic assay (D). Data shown are representative of 3 independent experiments.

ent agonists. fMLF and C5a desensitize the calcium responses to each other and to IL-8[56,57]. Desensitization of the calcium mobilization induced by SMC by preincubation with fMLF, IL-8, and LTB_4 indicates that chemotactic agents and SMC share common signaling pathways. This conclusion is further supported by the pertussis toxin sensitivity and PP2 insensitivity of the calcium-mobilizing activities of chemotactic factors and of SMC.

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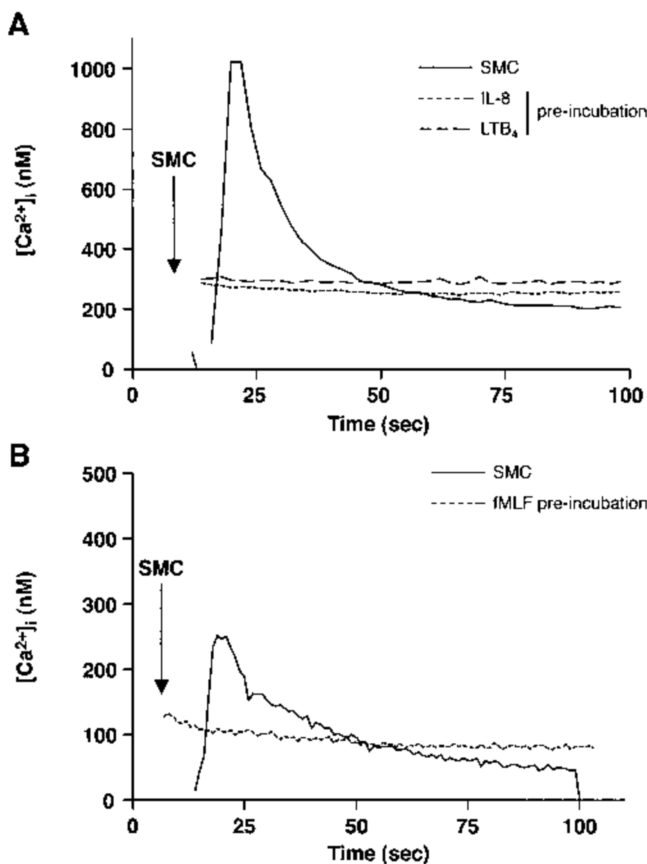

Figure 6. Cross-desensitization of the calcium mobilization response between IL-8, LTB_4, or fMLF and SMC. Neutrophils (10^7 cells/ml) were loaded with 1 μM Fura-2/AM as described in Materials and Methods, then stimulated with IL-8 (10^{-7} M) or LTB_4 (10^{-7} M) (A), or fMLF (10^{-7} M) (B) for 2 min before stimulation with 1 ml SMC (upper panel). Data shown are representative of 3 independent experiments.

Several inflammatory mediators are liberated by neutrophils in gouty arthritis. Lipid mediators[16,58], IL-1[14,15] and IL-8[59], oxygen radicals[60], S100A8/S100A9[61], and lysosomal enzymes[62] are all produced by neutrophils in response to MSU crystals. In gouty joints, there is also activation of complement[63]. Although many mediators have been identified in gouty arthritis, none of them, individually, is presently thought to be responsible for the bulk of the responses to MSU crystals. The lack of effect of the potent LTB_4 and PAF antagonists, of a cytosolic phospholipase A_2 inhibitor, and of an anti-IL-8 antibody on the activities of the SMC similarly indicates that the formation and secretion of these mediators do not account for the neutrophil-stimulating potential of the SMC.

It was also conceivable that the release of molecules such as formylated mitochondrial peptides following neutrophil lysis and necrosis may be responsible for the activity of SMC. However, previous studies have determined that very little neutrophil lysis was detected after interaction with MSU crystals[22,61]. Further, our data show that responses such as chemotaxis and calcium mobilization were not inhibited by incubation with cyclosporin H, an antagonist of the formyl peptide receptor[50], which eliminates endogenous formylated peptide signaling through the formyl peptide receptor as candidate for the activity in SMC. Preliminary data have shown that neutrophil degranulation can reproduce and desensitize the mobilization of calcium induced by the SMC. Cathepsin G, a granule protein, has previously been shown to be a chemoattractant for monocytes and to act through the formyl peptide receptor[49,64]. It could therefore have been responsible for the chemotactic activity of the SMC. However, cyclosporin H, which inhibits the activity of cathepsin G[49], did not affect the recruitment of neutrophils or the calcium mobilization induced by SMC. Studies are under way to characterize the role of granule components in the SMC.

The Centricon preparations are not reliable enough to give us a precise molecular weight, but they do nevertheless give some indications of the apparent size of the activation factor in the SMC. The chemotactic activity was mostly retained in the fractions above 30 kDa and below 50 kDa and none was found above 100 kDa. Lipid mediators, which have a very small molecular weight (< 1 kDa), are unlikely to be found above 30 kDa. Most obvious chemotactic agents have molecular weights averaging 10 kDa, including IL-8 (8–12 kDa, depending on its level of glycosylation) and S100 proteins. The activity in the SMC may derive from the association of multiple molecules into a complex that would not be separated under the native conditions used in our experiments.

A crystal chemotactic factor (CCF) with an estimated molecular weight of 15 kDa produced by neutrophils in response to MSU crystals has been described[25-27]. This factor is chemotactic for neutrophils and is produced after 1 hour[25–27], although shorter time periods were not discussed. CCF is not found preformed in neutrophils but is newly synthesized in response to MSU crystals[25]. Our own studies show some similarities between the activity observed and what has been associated with the CCF, although the activity was seen after only 5–15 minutes in the case of the SMC, instead of 1 hour as observed for CCF. The proposed molecular weight of CCF of 15 kDa does not correspond to our own indications of a molecular weight above 30 kDa. Further, the production of CCF was inhibited by cycloheximide and we found only a small inhibition of SMC production with cycloheximide within 15 minutes. Thus, it is unlikely that CCF accounts for a significant fraction of the activity of SMC.

The S100 family of proteins (also known as myeloid related proteins and calprotectin), particularly S100A8 and S100A9, are present in great quantities in neutrophils, are secreted in response to MSU crystals, and are chemotactic for neutrophils[3,61,65,66]. Human S100A12 showed strong chemotactic activity for monocytes and only weak chemotactic activity for neutrophils[67]. Several of our observations indicate that S100 proteins are not responsible for the neutrophil-activating properties of our SMC. The recovery of the activity of the SMC in fractions of Centricon separations larger than 30

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kDa argues against monomeric S100 proteins (with molecular weight < 10 kDa) being primarily responsible. The activity of the SMC was not inactivated by heat (data not shown), in contrast to that of recombinant S100A8/S100A9 proteins, which is lost upon heating[3]. The liberation of S100A8 and S100A9 induced by MSU crystals was inhibited by cytochalasin B[61], while generation of activity in the SMC was still observed in the presence of cytochalasin B (data not shown). Additionally, blocking antibodies for S100A8 and S100A9 did not inhibit the chemotactic responses or the mobilization of calcium observed in response to SMC (data not shown). Together, these results indicate that the activity observed in the SMC is unlikely to be due to S100A8, S100A9, or S100A8/S100A9.

Our study demonstrates that a chemotactic factor is released by neutrophils within 5 to 15 minutes of incubation with MSU crystals. This factor may be responsible for some of the neutrophil recruitment and activation in early phases of the inflammatory response in gout attacks. In addition to its chemotactic potential, the factor also elicits a mobilization of intracellular calcium and an increase in tyrosine phosphorylation in human neutrophils. These responses resemble those induced by classic chemotactic agents, a conclusion that is supported by the sensitivity of the responses to SMC to pertussis toxin. The liberation of an activation factor by neutrophils might serve to modulate neutrophil responses during the initial stage of inflammation, especially since this activation can be reproduced by inducing neutrophil degranulation. Special attention should be paid to determining the potential effects of SMC on inflammatory cells other than neutrophils. Further characterization and purification studies are under way to identify the factor and to determine its role in gout in particular and in inflammatory processes in general.

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