Crystal-induced Neutrophil Activation

IV. Specific Inhibition of Tyrosine Phosphorylation by Colchicine

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Abstract

We recently demonstrated that pathologically relevant inflammatory microcrystals, namely triclinic monosodium urate (MSU) and calcium pyrophosphate dihydrate (CPPD) crystals, potently stimulate a characteristic protein tyrosine phosphorylation pattern in human neutrophils that differed from that observed in response to other soluble or particulate agonists. In this study, the effects of colchicine on protein tyrosine phosphorylation induced by MSU and CPPD crystals in human blood neutrophils were investigated. Immunoblot analysis with antiphosphotyrosine antibodies demonstrated that colchicine dose-dependently inhibited the tyrosine phosphorylation of all the proteins phosphorylated in response to MSU and CPPD crystals. Other microtubule-disruptive agents such as vinblastine, nocodazole, and colcemid also inhibited crystal-induced protein tyrosine phosphorylation while lumicolchicine and trimethylcolchicinic acid were without effect. Indomethacin and phenylbutazone were similarly without effect on microcrystal-induced tyrosine phosphorylation. Colchicine, as well as the other active alkaloids, failed to inhibit the protein tyrosine phosphorylation elicited by FMLP, C5a, leukotriene B_4, and unopsonized zymosan. Overall, these results demonstrate that colchicine specifically and significantly inhibits the protein tyrosine phosphorylation induced by MSU and CPPD crystals and suggest that its effects are associated, at least in part, with its interaction with microtubules. Furthermore, the use of microtubule-disrupting drugs demonstrate that the mechanisms implicated in the induction of protein tyrosine phosphorylation by microcrystals differed from those involved in response to other soluble or particulate agonists. (*J. Clin. Invest.* 1993. 92:1722–1729.) Key words: arthritis • inflammation • gout • pseudogout • leukocytes • microtubules

Introduction

The deposition of several distinct microcrystals has been associated with the pathogenesis of acute and chronic articular syndromes. For example, it is now well established that the presence of monosodium urate (MSU)[1] and calcium pyrophosphate dihydrate (CPPD) crystals in joint diseases plays an important role in the development of gouty arthritis and joint chondrocalcinosis, respectively (1, 2). Although many cell types are involved in the pathogenesis of these inflammatory joint diseases, polymorphonuclear neutrophils play a central role particularly during the acute phase of the arthritis. Indeed, the inflammatory episodes observed in natural gout or in acute crystal-induced synovitis have been associated with a major accumulation of neutrophils in both the synovium and the synovial fluid, and many of these cells encounter and characteristically phagocytose crystals (2, 3). In addition, crystal-induced experimental arthritis in dogs is markedly suppressed when neutrophils are depleted by antipolymorphonuclear leukocyte serum or cytotoxic drugs (4, 5).

The activation of neutrophils by MSU and/or CPPD crystals leads to the production and secretion of several inflammatory mediators such as lysosomal enzymes (6, 7), oxygen-derived free radicals (8, 9), 5-lipoxygenase products (10), crystal-induced chemotactic factor (11, 12), and IL-1 (13). These mediators may be responsible, at least in part, for the systemic manifestations associated with crystal-induced joint disorders. The signaling pathway(s) involved in the mediation of these responses to microcrystals have only recently begun to be investigated. The addition of MSU and/or CPPD crystals to a suspension of human neutrophils leads to rapid increases in the cytoplasmic concentration of free calcium (10, 14), to the formation of inositol 1,4,5 triphosphate (14), to the activation of a phosphatidylcholine-specific phospholipase D (15), and to increases in the level of protein tyrosine phosphorylation (16). Two sets of results indicate that significant differences underlie the interaction of neutrophils with chemotactic factors and MSU or CPPD crystals. First, and somewhat indirectly, the responses to microcrystals have been found to be significantly more resistant to pertussis toxin than those of chemotactic factors (10, 17, 18), thereby implying the utilization of different coupling systems. Second, the tyrosine phosphorylation pattern stimulated by microcrystals was observed to be characteristic of these agonists and to differ qualitatively as well as quantitatively from that stimulated by a variety of soluble and particulate stimuli (16).

Among the therapeutic agents used in the treatment of acute arthritis, the usefulness of colchicine is relatively, although not exclusively, limited to crystal-associated rheumatic diseases such as gout (19). Although the effectiveness of colchicine has been reported as early as the sixth century (20), its precise mechanism of action is still obscure. Colchicine is thought to interfere with numerous neutrophil functions such

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1. *Abbreviations used in this paper:* CPPD, calcium pyrophosphate dihydrate; LTB_4, leukotriene B_4; MSU, monosodium urate.

as chemotaxis, adhesiveness, degranulation, and chemotactic factor release through its destabilizing action on microtubules (21–24). However, colchicine also binds proteins other than tubulin (25–27) and has effects on certain cell functions that are not related to the disruption of microtubules such as inhibition of nucleoside transport through the plasma membrane (28), suppression of phosphatidylcholine and cholesterol synthesis (29–31), inhibition of histamine (29), insulin (30), and parathormone (31) release, of platelet aggregation (32), and urate phagocytosis (33).

The present study was aimed at the examination of the possible impact of colchicine on an early intracellular event with signaling potential induced by microcrystals in human neutrophils, namely tyrosine phosphorylation. The specificity of colchicine's action on crystal-induced tyrosine phosphorylation was determined by assessing its effect on protein tyrosine phosphorylation induced by other soluble (FMLP, C5a, and leukotriene B_4 [LTB_4]) or particulate agonists (unopsonized zymosan). Microtubule-inactive colchicine analogues (lumicolchicine and trimethylcolchicinic acid) and other microtubule-disruptive agents (vinblastine, nocodazole, and colcemid) were also tested to determine the possible site of action of colchicine.

The results obtained demonstrate (*a*) that colchicine specifically inhibits, at least in part through its action on microtubules, crystal-induced tyrosine phosphorylation in human neutrophils, and (*b*) that the mechanisms implicated in the induction of protein tyrosine phosphorylation by microcrystals differ from those involved in response to other soluble or particulate agonists.

Methods

Compounds and reagents. Ficoll-Paque and Dextran T-500 were from Pharmacia (Dorval, Québec, Canada). HBSS and RPMI 1640 were from Gibco Labs (Grand Island, NY). FMLP, zymosan, colchicine, lumicolchicine, trimethylcolchicinic acid, vinblastine, nocodazole, and colcemid were obtained from Sigma Chemical Co. (St. Louis, MO). LTB_4 and biosynthetic recombinant C5a were generous gifts from Dr. R. Young (Merck-Frosst, Dorval, Québec, Canada) and H. J. Showell (Pfizer Central Research, Groton, CT), respectively. All stock solutions were prepared in low-endotoxin DMSO (Sigma Chemical Co.) and diluted in the incubation medium. The monoclonal antiphosphotyrosine antibody UB 05-321 and the horseradish peroxidase–labeled sheep anti–mouse IgG were purchased from UBI (Lake Placid, NY). The enhanced chemiluminescence Western blotting system was acquired from Amersham Corp. (Arlington Heights, IL).

Purification of neutrophils. Venous blood from healthy volunteers was sterilely collected on citrate/phosphate/dextrose/adenine anticoagulant solution. Neutrophils were isolated by means of 6% dextran sedimentation followed by standard techniques of Ficoll-Paque gradients and hypotonic lysis of erythrocytes. Neutrophils were resuspended in RPMI 1640, pH 7.4, at a final concentration of 15×10^6 cells/ml. Final cell preparations contained ≥ 98% neutrophils and cell viability exceeded 97% as measured by the release of lactate dehydrogenase activity.

Immunoblotting with antiphosphotyrosine antibodies. Freshly purified human neutrophils were incubated at 15×10^6 cells/ml with or without drugs for varying periods of time at 37°C before stimulation with the different agonists. The reaction was terminated by adding 100 μl of cell suspensions to 100 μl of boiling sample buffer (Tris-HCl, pH 6.8, 2 mM Na orthovanadate, 10 mM nitrophosphate, 10 mM NaF, 10 mM pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 20% SDS, 10% mercaptoethanol, 17.5% glycerol, and 0.1% bromophenol blue). The samples were immediately denatured by boiling for 7 min and loaded onto a 7.5–20% SDS polyacrylamide gel. After electrophoresis, the samples together with the molecular weight markers (Sigma Chemical Co.) were transfered onto Immobilon PDVF membranes (Millipore Corp., Bedford, MA) with the use of electrophoretic transfer cells (Hoeffer Scientific Instruments, Canberra Packard, Ontario, Canada). The blots were then stained with Ponceau S to locate the molecular weight markers on the blots. Nonspecific sites were blocked using 2% gelatin in TBS-Tween 0.1% (25 mM Tris-HCl, pH 8.0, 190 mM NaCl, 0.1% [vol/vol] Tween 20) for 1 h at 37°C. The monoclonal antibody UB 05-321 was then incubated with the membranes for 1 h at 37°C at a final dilution of 1:4,000 in 2% gelatin, 0.1% TBS-Tween. The membranes were washed at room temperature three times in TBS-Tween 0.1% and further incubated with horseradish peroxidase–labeled sheep anti–mouse IgG for 45 min at 37°C at a final dilution of 1:20,000 in 2% gelatin, 0.1% TBS-Tween. The membranes were then washed three times as described above and covered with the enhanced chemiluminescence detection solution according to the manufacturer's instructions. The phosphotyrosine bands were then visualized by autoradiography after exposure to Kodak X-Omat films. Selected autoradiograms were analyzed using a densitometer (Research Analysis System, Amersham Canada, Oakville, Ontario, Canada), and the values were corrected for background and expressed as arbitrary units.

Preparation of microcrystals. MSU and CPPD microcrystals were prepared by modifications of previously described methods (34–36). Briefly, a boiling MSU solution (0.03 M, pH 7.5) was prepared by dissolution of equimolar quantities of uric acid and sodium hydroxide and filtered on an Acropor membrane filter (AN-3000, 3 μM; Gelman Sciences, Inc., Ann Arbor, MI). Sodium chloride (0.1 M final concentration) was added to speed up and improve the uniformity of the crystallization. CPPD was obtained by mixing a calcium nitrate solution (0.1 M final concentration) with an acidic solution of sodium pyrophosphate (final concentration 0.025 M of $Na_2P_2O_7$ and 0.03 M HNO_3). The milky-white precipitate formed CPPD crystals after a 1-d incubation at 50–60°C. The crystals were characterized by x-ray diffraction (Rigaku Geigerflex D/max), by examination under phase and polarizing microscopy and by scanning electron microscopy. The MSU and CPPD crystals showed triclinic morphologic characteristics. Their dimensions as determined by scanning microscopy were $10 \times 1 \times 1$ μm to $25 \times 1.5 \times 1.5$ μm and $12 \times 1.4 \times 1.4$ μm to $25 \times 1.7 \times 1.7$ μm for MSU and CPPD, respectively. MSU and CPPD preparations were free of endotoxins (as assessed by the Limulus Assay, Whittaker, Walkersville, MD) and used without opsonization.

Statistical analysis. Results from densitometric analysis of autoradiograms are expressed as mean±SEM. Statistical analysis was performed by Student's paired *t* test (two-tailed), and significance was considered attained when P was ≤ 0.05.

Results

Effects of colchicine on stimulated tyrosine phosphorylation in human neutrophils. Neutrophils were preincubated with either diluent (DMSO) or 10 μM colchicine for 1 h at 37°C. The cells were then stimulated with or without 3 mg/ml MSU or CPPD crystals for 30 and 45 min, respectively, as previously determined to be optimal (16). An immunoblot representative of six different individual experiments is shown in Fig. 1. Only low levels of tyrosine phosphorylation were observed for nonstimulated neutrophils. A doublet of an approximative molecular mass of 55 kD was constitutively tyrosine phosphorylated, and its intensity was found not to be altered under any of the conditions tested. The addition of MSU or CPPD crystals to diluent-treated neutrophils consistently induced the tyrosine phosphorylation of at least five proteins with apparent molecular masses of 130, 118, 80, 70, and 60 kD, the 70-kD protein

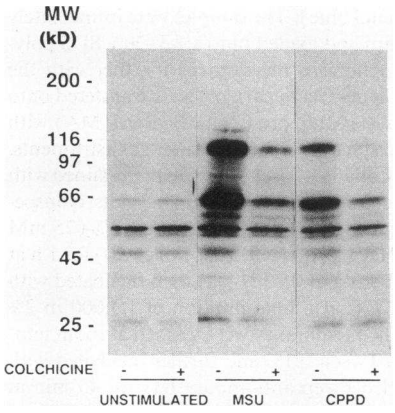

Figure 1. Effect of colchicine on microcrystal-induced tyrosine phosphorylation. Neutrophils (15×10^6/ml) were preincubated with diluent (DMSO) or with 10 μM colchicine for 1 h at 37°C. The cells were further incubated with 3 mg/ml of MSU or CPPD crystals for 30 and 45 min, respectively. Blotting and revelation of the phosphotyrosine bands were carried out as described in Methods. The immunoblot shown is representative of six different individual experiments.

(or proteins) showing the greatest level of tyrosine phosphorylation (Figs. 1 and 2). Preincubation of human neutrophils with colchicine led to a strong inhibition of the tyrosine phosphorylation induced in response to MSU and CPPD crystals (Figs. 1 and 2). The autoradiograms of these six different experiments were selectively analyzed, and the densitometric analysis of the two major bands (pp70 and pp118) demonstrated that MSU- and CPPD-induced tyrosine phosphorylation of these proteins was significantly inhibited by 62–73% (Fig. 2). Colchicine also significantly reduced the phosphorylation of the 60- and 130-kD proteins by 59–75% (data not shown). Colchicine, by itself, increased to a small extent the phosphorylation level of the 60-, 70-, and 118-kD proteins in unstimulated cells. The effect of colchicine on microcrystal-induced protein tyrosine phosphorylation was dose dependent, being detectable at concentrations as low as 10^{-7} M and optimal at 10^{-5} M (data not shown).

The specificity of colchicine's effects on the stimulation of tyrosine phosphorylation in human neutrophils was determined next using agonists other than crystals. Neutrophils were preincubated for 1 h with either DMSO or 10 μM colchicine, and further stimulated for 1 min with 100 nM fMLP, C5a, or LTB_4 or for 30 min with 3 mg/ml unopsonized zymosan (Fig. 3). These times were previously determined to be optimal for the various stimuli. Although these agonists stimulated the tyrosine phosphorylation of a set of proteins that overlapped (within the limits of resolution of a one-dimensional analysis) with that detected with the microcrystals, important qualitative differences were however noted. The major, indeed diagnostic, differences between these agonists being the predominance of the 118-kD band in case of unopsonized zymosan and the soluble agonists, and of the 70-kD band in the case of the microcrystals. Pretreatment of human neutrophils with colchicine was without any effect on the phosphorylation of the 118-kD substrate induced by any of these agonists (Fig. 4). Densitometric analysis of protein tyrosine phosphorylation induced by FMLP, C5a, or LTB_4 showed that colchicine caused a 2.0-, 3.1-, and 3.2-fold increase in the phosphorylation of the 60-kD (data not shown) and 4.0-, 12-, and 8-fold increase of the 70-kD (Figs. 3 and 4) proteins, respectively.

Effects of other microtubule-disruptive agents on crystal-induced tyrosine phosphorylation. To determine if the inhibitory effects of colchicine on crystal-induced tyrosine phosphorylation were related to its action on microtubules, the effects of other microtubule-disruptive agents were investigated. Neutrophils were preincubated with either DMSO or with 10 μM vinblastine, nocodazole, or colcemid for 1 h at 37°C and then stimulated with 3 mg/ml MSU or CPPD crystals for 30 and 45 min, respectively. Vinblastine, nocodazole, and colcemid reduced the tyrosine phosphorylation of all the proteins stimulated in response to MSU and CPPD crystals (Fig. 5). In contrast, vinblastine and nocodazole failed to inhibit the tyrosine phosphorylation induced by FMLP (Fig. 5), C5a, LTB_4, or unopsonized zymosan (Fig. 6). In addition, colcemid, like colchicine, increased the tyrosine phosphorylation of the 60- and 70-kD proteins in response to all the soluble agonists tested, while unopsonized zymosan-induced tyrosine phosphoryla-

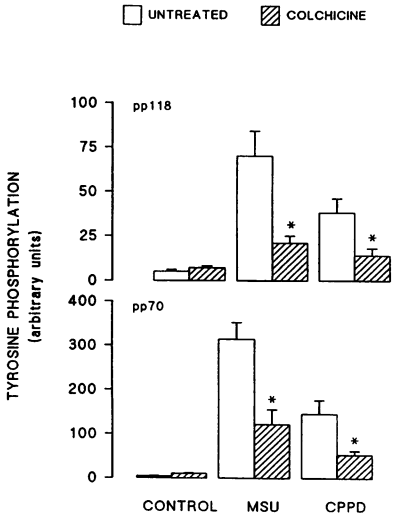

Figure 2. Quantification of the effects of colchicine on crystal-induced tyrosine phosphorylation in human neutrophils. Neutrophils (15×10^6/ml) were treated with diluent (DMSO) or with 10 μM colchicine for 1 h at 37°C and further incubated with 3 mg/ml MSU or CPPD crystals for 30 and 45 min, respectively. Blotting and revelation of the phosphotyrosine bands were carried out as described in Methods. The autoradiograms from six different experiments were selectively analyzed for the major tyrosine-phosphorylated proteins, pp70 and pp118, using a Research Analysis System densitometer. The values were corrected for background and expressed as arbitrary units (mean±SEM). Values significantly different from appropriate control are indicated by $*P \leq 0.05$.

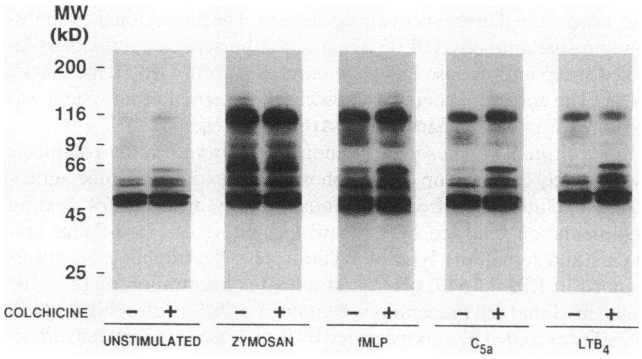

Figure 3. Effect of colchicine on protein tyrosine phosphorylation induced by unopsonized zymosan, FMLP, C5a, and LTB_4. Neutrophils (15×10^6/ml) were preincubated with diluent (DMSO) or with 10 μM colchicine for 1 h at 37°C and further incubated with 3 mg/ml unopsonized zymosan for 30 min or 100 nM FMLP, C5a, or LTB_4 for 1 min. Blotting and revelation of the phosphotyrosine bands were carried out as described in Methods. The immunoblot shown is representative of three different individual experiments.

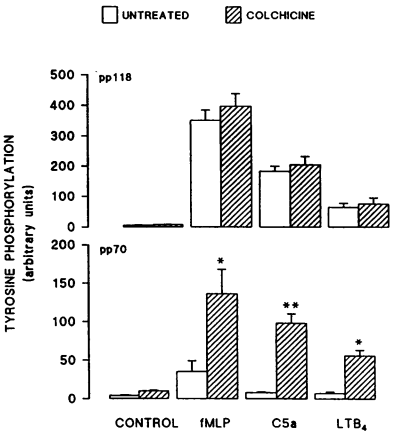

Figure 4. Quantification of the effect of colchicine on protein tyrosine phosphorylation induced by FMLP, C5a, and LTB_4. Neutrophils (15×10^6/ml) were treated with diluent (DMSO) or with 10 μM colchicine for 1 h at 37°C and further incubated with 100 nM FMLP, C5a, or LTB_4 for 1 min. Blotting and revelation of the phosphotyrosine bands were carried out as described in Methods. The autoradiograms from four different experiments were selectively analyzed for the major tyrosine-phosphorylated proteins, pp70 and pp118, using a Research Analysis System densitometer. The values were corrected for background and expressed as arbitrary units (mean±SEM). Values significantly different from appropriate control are indicated by $*P \leq 0.05$ and $**P \leq 0.01$.

tion was affected only to a small extent, if at all (Fig. 6). Colcemid, by itself, increased to a small extent the phosphorylation levels of the 60-, 70-, and 118-kD proteins in control cells (Fig. 5).

Effect of colchicine analogues on crystal-induced tyrosine phosphorylation in human neutrophils. Lumicolchicine and trimethylcolchicinic acid, two colchicine analogues that neither bind tubulin nor disrupt the microtubule network were studied next. Neutrophils were preincubated in the presence of 10 μM lumicolchicine or trimethylcolchicinic acid for 1 h at 37°C and further stimulated with 3 mg/ml MSU or CPPD crystals for 30 and 45 min, respectively. Neither lumicolchicine nor trimethylcolchicinic acid inhibited the microcrystal- (Fig. 7) or the soluble agonist- or unopsonized zymosan–induced tyrosine phosphorylation (data not shown). Furthermore, neither compounds enhanced the phosphorylation of the 60- and 70-kD bands in response to FMLP, C5a, or LTB_4 (data not shown).

Effects of phenylbutazone and indomethacin on crystal-induced tyrosine phosphorylation. Phenylbutazone and indomethacin, two nonsteroidal anti-inflammatory agents, have previously been described as being effective inhibitors of certain neutrophils functions triggered by microcrystals. Indeed, both drugs block the phagocytosis of CPPD crystals, indirectly lessening the release of cell-derived chemotactic factor (37). Neither compound inhibited to a significant degree the stimulation of tyrosine phosphorylation induced by MSU or CPPD crystals (Table I). Colchicine, on the other hand, did produce its characteristic inhibitory effects in the same experiments (data not shown).

Discussion

Numerous soluble agonists such as granulocyte-macrophage colony-stimulating factor (38), C5a, FMLP (39–42), LTB_4 (42), platelet-activating factor (43), heat-aggregated IgG (44), and phorbol esters (41, 42) have been shown to induce a rapid

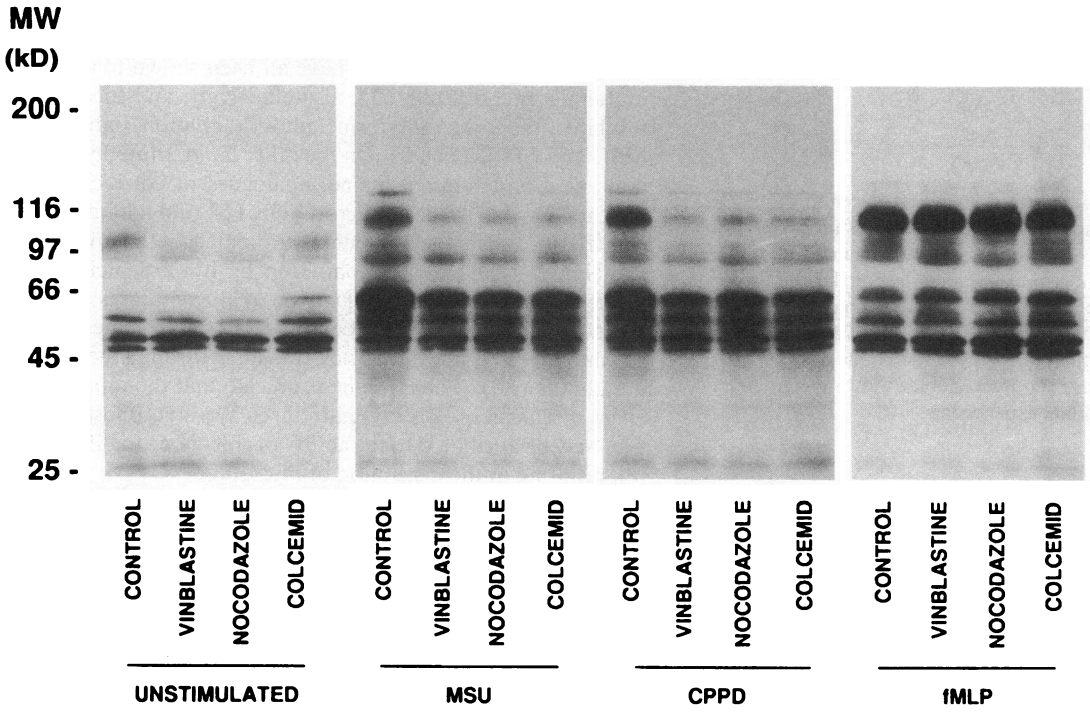

Figure 5. Effect of vinblastine, nocodazole, and colcemid on microcrystal- and FMLP-induced tyrosine phosphorylation. Neutrophils (15×10^6/ml) were preincubated with diluent (DMSO) or with 10 μM vinblastine, nocodazole, or colcemid for 1 h at 37°C and further incubated with 3 mg/ml of MSU or CPPD crystals for 30 and 45 min, respectively, or with 10^{-7} M FMLP for 1 min. Blotting and revelation of the phosphotyrosine bands were carried out as described in Methods. The immunoblot shown is representative of three different individual experiments.

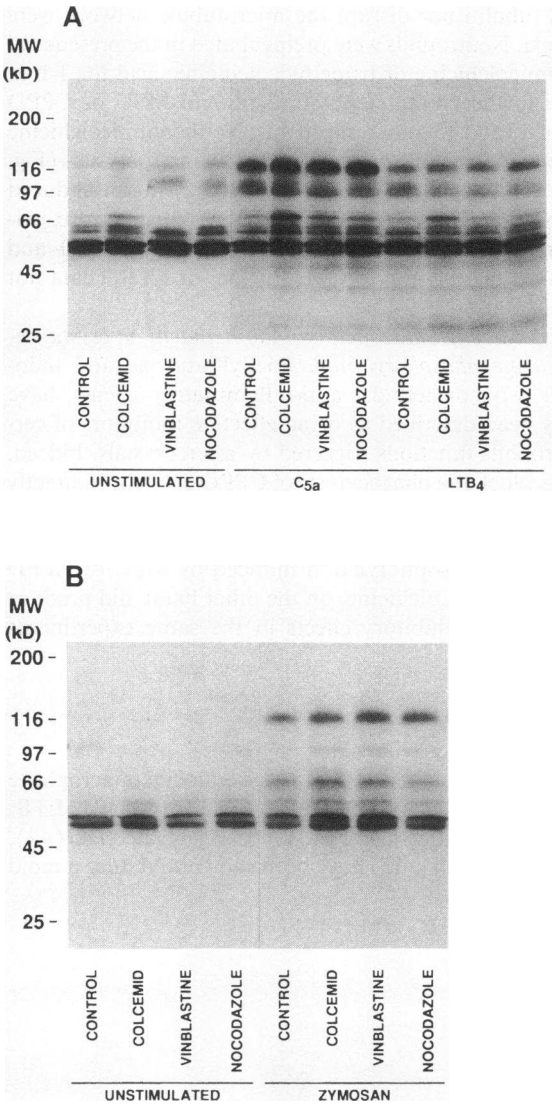

Figure 6. Effect of vinblastine, nocodazole, and colcemid on protein tyrosine phosphorylation induced by C5a, LTB_4, and unopsonized zymosan. Neutrophils (15×10^6/ml) were preincubated with diluent (DMSO) or with 10 μM vinblastine, nocodazole, or colcemid for 1 h at 37°C and further incubated with 3 mg/ml of unopsonized zymosan for 30 min or with 100 nM FMLP, C5a, or LTB_4 for 1 min. Blotting and revelation of the phosphotyrosine bands were carried out as described in Methods. The immunoblot shown is representative of three different individual experiments.

phosphorylation on tyrosine residues of several protein substrates. The tyrosine phosphorylation profiles induced by these soluble agonists are quite similar, the major tyrosine phosphorylated substrate(s) having an approximate molecular mass of 118-kD. We recently demonstrated that MSU and CPPD crystals potently stimulate a characteristic protein tyrosine phosphorylation pattern in human neutrophils that differed from that observed in response to other soluble or particulate agonists (16). In contrast to soluble agonists that stimulate predominantly pp118, a protein with an apparent molecular mass of 70-kD showed the greatest phosphotyrosine accumulation in response to these two crystals. The results of the present study demonstrate that colchicine specifically inhibits the protein tyrosine phosphorylation stimulated by MSU and CPPD crystals but not by the other agonists (soluble or particulate) and suggest that the effects of the alkaloid are mediated by its disruptive action on microtubules. In so doing, this study underlines the relevance of the studies aimed at the elucidation of the transductional pathways mediating the interaction of microcrystals with phagocytes to the eventual understanding of the phlogistic potential of these stimuli.

Among the therapeutic agents used in the prevention and treatment of acute arthritis, colchicine is unique in that its usefulness is generally felt to be relatively limited to crystal-associated rheumatic diseases such as gout and pseudogout (19) and to be dependent on its early administration. The present results demonstrate that the alkaloid specifically inhibits one of the potentially critical biochemical responses initiated by crystals in human neutrophils, namely protein tyrosine phosphorylation. Colchicine significantly inhibited the protein tyrosine phosphorylated in response to both MSU and CPPD microcrystals at concentrations that are of the same order of magnitude as those that have been measured in the serum and peripheral blood leukocytes of patients after intravenous injection of usual therapeutic doses of colchicine, i.e., 10^{-7}–10^{-6} M (45, 46).

The present results also indicate that colchicine most likely alters crystal-induced protein tyrosine phosphorylation through its effects on tubulin and/or microtubule functions. The concentration and preincubation times required for colchicine to inhibit crystal-induced protein tyrosine phosphorylation are similar to those that have been shown to be optimal for the inhibition of the assembly of microtubules in neutrophils (47, 48) and for the promotion of concanavalin A cap formation in human polymorphonuclear leukocytes (49–51), a response that is thought to be dependent on microtubule disassembly. Other synthetic drugs such as vinblastine, nocodazole, and colcemid, which have all been shown to alter microtubule integrity (52, 53), also effectively, and specifically, inhibited the protein tyrosine phosphorylation induced by MSU and CPPD (Fig. 5). On the other hand, trimethylcolchicinic acid and lumicolchicine, analogues of colchicine that have no known effects on microtubules (54) did not inhibit the protein tyrosine phosphorylation stimulated by the microcrystals (Fig. 7). The lack of inhibition by lumicolchicine is of particular interest as this colchicine analogue shares some of the non-microtubule–dependent effects of colchicine such as the interference with nucleoside transport through the plasma membrane (28). These findings indicate that the modulatory effect of colchicine on crystal-induced tyrosine phosphorylation may be attributed to the ability of colchicine to bind tubulin, resulting in the disruption of microtubules.

The responses of neutrophils to microcrystals may result from direct physical contacts with the plasma membrane and/or from the subsequent internalization of the particulate stimuli. The microscopic observation of neutrophils in crystal-induced synovial fluids does not allow a distinction between these alternative (or complementary) mechanisms. Although neutrophils phagocytosing crystals can indeed be demonstrated in inflammatory exudates, it is also commonly observed that a large percentage of the cells in the synovial fluids are free of microcrystals. Several lines of evidence suggest that the stimulation of tyrosine phosphorylation by MSU and CPPD crystals is not critically dependent on particule engulfment and that the actions of colchicine on the crystal-induced

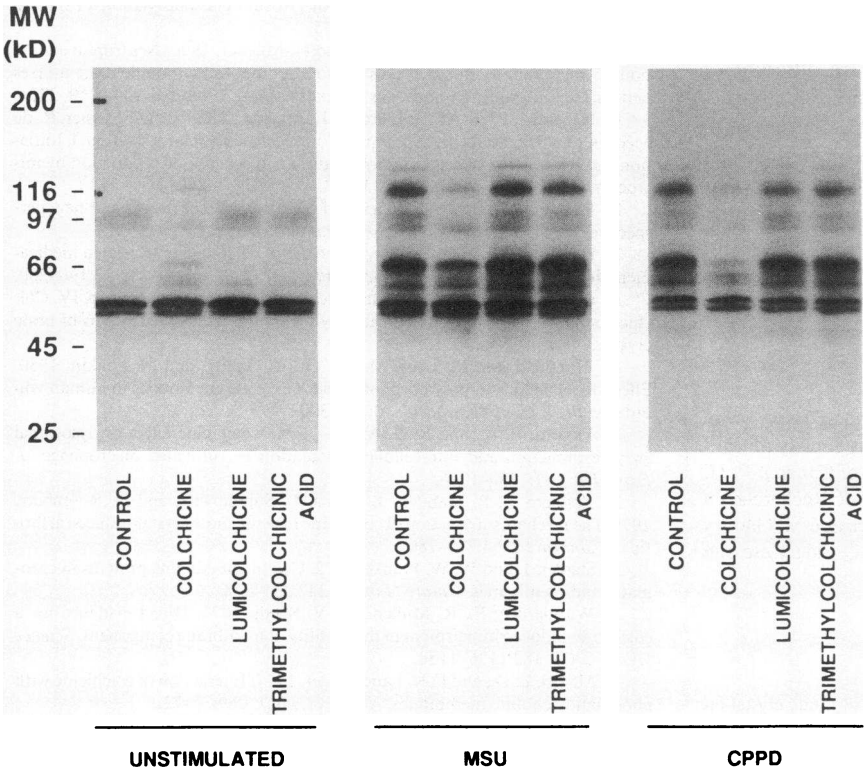

Figure 7. Effect of lumicolchicine and trimethylcolchicine acid on microcrystal-induced tyrosine phosphorylation. Neutrophils (15×10^6/ml) were preincubated with diluent (DMSO) or with 10 μM lumicolchicine or trimethylcolchicinic acid at 37°C for 1 h further incubated with 3 mg/ml of MSU or CPPD crystals for 30 and 45 min, respectively, or with 100 nM FMLP for 1 min. Blotting and revelation of the phosphotyrosine bands were carried out as described in Methods. The immunoblot shown is representative of three different individual experiments.

tyrosine phosphorylation do not result from a suppression of phagocytosis. The tyrosine phosphorylation elicited by microcrystals does not appear to be a generalized response to the phagocytic process as the responses to other particulate stimuli such as unopsonized zymosan or latex beads (16) and heat-killed staphylococcus or silica crystals (Roberge, C. J., unpublished results) are weaker and qualitatively different from those of MSU or CPPD crystals (16). In addition, colchicine failed to inhibit zymosan-induced tyrosine phosphorylation (Fig. 3). Furthermore, nonsteroidal anti-inflammatory drugs such as phenylbutazone and indomethacin, which block CPPD crystal and starch granule phagocytosis by neutrophils (55, 56), did not affect crystal-induced tyrosine phosphorylation (Table I).

Table I. Effects of Indomethacin and Phenylbutazone on Crystal-induced Tyrosine Phosphorylation

	Level of tyrosine phosphorylation			
	pp60	pp70	pp118	pp130
	Arbitrary scanning units			
Control	34±18[1]	6±3	9±4	1±1
Indomethacin	53±25	11±7	16±7	2±1
Phenylbutazone	49±24	11±6	13±6	2±1
MSU	158±60	328±97	61±16	22±4
Indomethacin/MSU	223±79	409±99	76±22	21±6
Phenylbutazone/MSU	193±56	371±68	80±16	22±3
CPPD	54±29	72±29	32±14	6±2
Indomethacin/CPPD	65±26	96±10	30±6	8±3
Phenylbutazone/CPPD	57±33	68±30	29±17	7±4

[1] Neutrophils (15×10^6/ml) were treated with diluent or with 10 μM phenylbutazone or indomethacin for 1 h at 37°C and further incubated with 3 mg/ml MSU or CPPD crystals for 30 and 45 min, respectively. Blotting and revelation of the phosphotyrosine bands were carried out as described in Methods. The autoradiograms from five different experiments were selectively analyzed for pp60, pp70, pp118, and pp130 using a Research Analysis System densitometer. The values were corrected for background and expressed as arbitrary units (mean±SEM).

The finding that the inhibitory effect of colchicine was limited to the protein tyrosine phosphorylation stimulated by MSU and CPPD crystals is of particular interest to the physiology of these cells. At the very least, these results demonstrate that the levels of tyrosine phosphorylation in human neutrophils can be modulated by various, and pharmacologically differentiable, mechanisms. More specifically, they suggest the involvement of distinct enzymatic pathways (tyrosine kinases and/or phosphatases) in the responses to inflammatory microcrystals and to the other agonists tested in the present study. The functional relevance of tyrosine phosphorylation in human neutrophils remains to be clearly defined. Among others, the nature of the effector pathways, the activity of which is modulated by tyrosine phosphorylation, remains to be identified. However, a pharmacological correlation has previously been drawn between the stimulation of tyrosine phosphorylation and of the oxidative burst (40, 57–59). The differential effect of colchicine on the stimulation of tyrosine phosphorylation and on the production of superoxide anions (16; Roberge, C. J., unpublished results) by microcrystals and soluble agonists reinforces this correlation.

Overall, the results presented in this study suggest a novel putative site of action for colchicine by demonstrating that the alkaloid can specifically inhibit one of the biochemical responses initiated by MSU and CPPD crystals in human neutrophils. The agonist-specificity of colchicine's action demonstrates that the mechanism of neutrophil activation by MSU and CPPD crystals differs from those triggered by soluble che-

motactic factors and formylated peptides. These findings suggest that the mechanisms implicated in the induction of protein tyrosine phosphorylation in neutrophils are different or differently regulated depending on the agonist used. An attractive hypothesis is that microcrystals activate a specific subset of kinases and/or phosphatases, directly or indirectly associated to tubulin or to the microtubule network, that would be responsible for the characteristic tyrosine phosphorylation pattern elicited by the microcrystals, and its specific modulation by colchicine. However, the precise mechanism whereby crystal-induced tyrosine phosphorylation in neutrophils is modulated by microtubules remains to be defined.

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