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Research Article

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Release of Platelet Constituents by Monosodium Urate Crystals

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ABSTRACT The release of human platelet constituents by the etiologic agent of gout, the monosodium urate crystal, is described here. In suspensions of washed platelets, response to urate crystals proceeded in two phases: A secretory phase involved the rapid active release of serotonin, ATP, and ADP with little loss of lactic dehydrogenase or β -glucuronidase. A lytic phase involved the slower loss of all platelet constituents.

Both phases were inhibited by iodoacetate plus dinitrophenol, suggesting an energy requirement. In ultrastructural studies, lysis of washed platelets which appeared to contain crystals was seen. Urate crystals were also shown to induce serotonin release and platelet lysis in citrated platelet-rich plasma.

Since urate crystals are deposited at a variety of sites, urate crystal-platelet interaction *in vivo* is a possibility. Such interactions, leading to release of platelet constituents, might contribute to gouty inflammation or to enhanced atherogenesis.

INTRODUCTION

Recent studies have suggested a role for platelets in inflammation by release of mediators such as vasoactive amines (1-3), chemotactic activity-generating factors (4), permeability-enhancing factors (5, 6), bactericidal proteins (7), and prostaglandin intermediates (8) during platelet activation. Monosodium urate crystals are particles deposited in diverse tissues in gouty individuals (9) where they might then come in contact with platelets and are the initiators of inflammation (10, 11)

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in this disease. Various other particulates stimulate platelets *in vitro* (12-21). This led us to ask, Does the activation of platelets play a role in gouty inflammation? As a first step in answering this question, we have studied the interaction between the particle found in human gout, i.e., microcrystalline monosodium urate, and human platelets. In this report we demonstrate urate crystal-induced platelet activation as assessed by serotonin release in citrated platelet-rich plasma and serotonin, ATP and ADP release, shape change, and lysis in suspensions of washed platelets.

METHODS

Chemicals

The following were obtained from Sigma Chemical Co., St. Louis, Mo.: ellagic acid, practical; firefly lantern extract (desiccated); iodoacetic acid; NADH (disodium salt grade II); phenolphthalein glucuronic acid (sodium salt); phosphoenolpyruvate (trisodium salt); sodium pyruvate; bovine serum albumin.

Uric acid was from ICN Nutritional Biochemicals Div., International Chemical & Nuclear Corp., Cleveland, Ohio. 2,4-Dinitrophenol (2,4-DNP)¹ was from Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y., and disodium EDTA from J. T. Baker Chemical Co., Phillipsburg, N. J.

Serotonin [2-¹⁴C]binoxalate in 2% ethanol, 27.5 mCi/mmol, was obtained from New England Nuclear, Boston, Mass., and was stored at 100 μ Ci/ml at 4°C. [³H]serotonin creatinine sulfate powder was from Amersham/Searle Corp., Arlington Heights, Ill., and was dissolved at 100 μ Ci/ml in 0.01 M phosphate buffer, pH 7, and stored in 0.5-ml aliquots at -40°C. [⁵¹Cr]sodium chromate (100 μ Ci/mg) was also from Amersham/Searle.

Crystals

Monosodium urate crystals were prepared by neutralization of thrice recrystallized uric acid (22) to form needle-shaped,

¹Abbreviations used in this paper: ACD, acid-citrate-dextrose; 2,4-DNP, 2,4 dinitrophenol; LDH, lactic dehydrogenase; PMN, polymorphonuclear leukocytes; PRP, platelet-rich plasma; TBS, Tris-buffered saline.

negatively birefringent crystals. All crystals were heated to 200°C for 2 h to remove possible pyrogens and suspended in 0.001 M phosphate-buffered 150 mM NaCl, pH 7.4, before use.

Platelet Preparations

All preparations were made in plastic apparatus.

Buffers. The following buffers were employed in platelet preparation: (a) Tris-buffered saline (TBS) was NaCl 7 g/liter, Tris 3.6 g/liter, glucose 1 g/liter, pH 7.4. (b) Tyrode's solution: NaCl 8 g/liter, KCl 0.195 g/liter, NaHCO₃ 1.02 g/liter, MgCl₂ 6H₂O 0.213 g/liter, CaCl₂ (anhydrous) 0.145 g/liter, glucose 1 g/liter, pH 7.4 (c) Tyrode's without calcium: the same as described in item (b), but with CaCl₂ omitted and pH adjusted to 6.5.

Platelet-rich plasma. Blood from normal donors who had taken no medication in the previous week was drawn through a 20-gauge needle into 0.1 vol 3.8% trisodium citrate, pH 7.4. Platelet-rich plasma (PRP) was isolated by centrifugation at 200 g in an International Centrifuge (International Equipment Co., Boston, Mass.) for 15 min at room temperature and was incubated with 10 μCi radioactive serotonin at 37°C for 30 min, resulting in uptake of 80–90% of radioactivity. Such "labeled PRP" contained 2–4 × 10⁸ platelets and 0–4 × 10⁵ white cells per milliliter.

Tris-buffered saline platelet suspensions. Labeled PRP was obtained as above, using 0.1 vol disodium EDTA, pH 7.4, as anticoagulant. Suspensions of washed platelets were prepared from this EDTA-PRP by centrifugation at 1,000 g at 8°C for 15 min in a Sorvall RC-2B centrifuge, resuspension in 40 ml TBS, pH 7.4, using a Schwarz Mann Biopipet (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.), recentrifugation, and resuspension twice more, before finally being suspended in the appropriate volume of TBS to yield platelet suspensions containing 0.8–3 × 10⁸ platelets/ml and 0–2 × 10⁵ white blood cells/ml.

Albumin density gradient washed platelets. These were prepared according to Walsh (23) from acid-citrate-dextrose (ACD) (24) anticoagulated labeled PRP with use of Tyrode's without Ca⁺⁺, pH 6.5, as buffer in all washes. After the third wash, the platelets were harvested with care to minimize inevitable albumin contamination and were suspended at 2 × 10⁸/ml in Tyrode's without Ca⁺⁺, pH 6.5. Immediately before use they were diluted 1:10 with protein-free Tyrode's solution.

Double labeled platelets. ACD PRP was centrifuged at 1,000 g for 15 min at 8°C and the platelet button suspended in 0.1 vol of plasma. 200 μCi ⁵¹Cr as sodium chromate and 20 μCi [¹⁴C]serotonin were added and the cells incubated at 37°C for 60 min. After three washes in albumin gradients prepared according to Walsh (23), as described above, the cells were suspended at 2 × 10⁸/ml in autologous citrated platelet-poor plasma.

Experiments

0.8 ml of "labeled PRP" or platelet suspension in polyethylene tubes was preincubated at 37°C for 10 min; then 0.2 ml of buffer or crystal suspension was added and the mixture incubated with continuous shaking at 37°C in a Dubnoff metabolic incubator. At appropriate times, tubes were placed into iced methanol for 2–5 min and subsequently centrifuged at 12,500 g at 0°C for 20 min. Supernates were removed for assays and 1 ml of buffer was added to the pellets, which were then sonicated at "7," 4 s, four times in a Sonifier (Bronwill Scientific, Rochester, N. Y.; model 1850) cell disrupter. The sonicate

was centrifuged at 12,500 g for 20 min and the platelet extract was taken for assays. Percent release of any component was defined as

$$\frac{\text{Activity in crystal-treated supernate} - \text{Activity in buffer-treated supernate}}{\text{Activity in buffer-treated pellet}} \times 100.$$

Less than 10% of total platelet radioactivity was noted in control supernates.

Polymorphonuclear leukocytes

Peripheral blood leukocytes, >80% polymorphonuclear leukocytes (PMN), were prepared from normal male donors and suspended in Krebs-Ringer phosphate, pH 7.4, as previously described (25).

Assays

Radioactivity was assayed by mixing 100 μl of sample with 900 μl of water and adding 10 ml of Bray's solution before counting in a Packard Tri-Carb liquid scintillation counter. (Packard Instrument Co., Inc., Downers Grove, Ill.).

Lactic dehydrogenase (LDH) was assayed by a standard technique (26) and β-glucuronidase by hydrolysis of phenolphthalein glucuronic acid as previously described.²

100 μl of ethanol-EDTA extracts of supernatants and platelet pellets were prepared for assay of ATP and ADP as recommended by Holmsen et al. (27). Light emission after addition of 500 μg of firefly lantern extract was measured in a Packard Tri-Carb liquid scintillation counter as suggested by Stanley and Williams (28).

Thin-layer chromatography

This was performed with precoated silica gel thin-layer chromatography plates (EM Laboratories, Inc., Elmsford, N. Y.) in an ascending ethyl acetate:isopropanol:NH₄OH (60:35:5) system which resolved a serotonin standard from 5-hydroxytryptophol and 5-hydroxy-indole acetic acid standards. The radioactivity migrating with 100 μg of carrier serotonin creatinine sulfate was determined as was the radioactivity on the remainder of the plate. Results were expressed as percent of total radioactivity migrating with the carrier serotonin.

Hageman factor activation

A crude estimate of Hageman factor activation was obtained by a standard partial thromboplastin time assay (29) using 2% Celite filter cel suspension (Johns-Manville, Denver, Colo.) rather than kaolin as a positive substance control. One-tenth of the material to be tested, or of Celite, was incubated with pooled normal plasma at 37°C for 4 min, 0.1 ml of 1 mg/ml Inosithin (Associated Concentrates, N. J.) and then 0.1 ml of 0.025 M CaCl₂ were added, and clotting time was determined in a Fibrometer (Standard Scientific, Chicago, Ill.).

Morphologic studies

Platelet suspensions at 2 × 10⁸ cells/ml were prepared in Tyrode's solution by the albumin density gradient technique

² Ginsberg, M. H., F. Kozin, J. May, D. Chow, and J. L. Skosey. 1977. The adsorption of polymorphonuclear leukocyte lysosomal enzyme activity to monosodium urate crystals. *Arthritis Rheum.* In press.

of Walsh (23) as described above or in Tris-buffered saline and glucose as for the release assays. Crystals, at a concentration of 2 mg/ml, were incubated with platelets in a volume of 500 μ l at 37°C with horizontal shaking. At 30 min, 3 ml of prewarmed (37°C) 2% glutaraldehyde in isotonic-buffered saline (30) was added, and this mixture was incubated at 37°C for 30 min and 4°C for 1 h. Washed cell pellets were then postfixed at 4°C in 1% osmium tetroxide in isotonic-buffered saline for 30 min and then dehydrated in graded alcohols, embedded in Epon, and sectioned with an LKB ultramicrotome (LKB Instruments, Inc., Rockville, Md.). Uranyl acetate and lead citrate-stained grids were examined in a Hitachi HU 11E electron microscope.

RESULTS

Effect of urate crystals on suspensions of washed platelets

Urate crystals induced serotonin release in suspensions of washed platelets, whereas addition of supersaturated urate solution to a final concentration of 6 mg/100 ml failed to induce release, indicating that the physical state of the crystals was important. That released radioactivity represented serotonin was next verified.

Identity of released serotonin. In this experiment 0.8 mg/ml urate crystals induced release of $76 \pm 5.5\%$ of radioactivity from [14 C]serotonin-labeled platelets at 10 min. In thin-layer chromatographic studies, $86.4 \pm 2.1\%$ (SE) of the released radioactivity was associated with the carrier serotonin standard. $85.4 \pm 2\%$ of radioactivity recovered from sonicated labeled platelets and $90 \pm 1.5\%$ of radioactivity released in the presence of 5 NIH Units/ml of crude bovine thrombin (Park, Davis & Co., Detroit, Mich.) (released $95 \pm 1.2\%$ of platelet radioactivity) migrated with a serotonin standard. Hence, there was little if any metabolism of serotonin induced by urate crystals.

Time course of urate crystal-induced release. The time course of release of platelet constituents in response to urate crystals appears in Fig. 1. Since proteins

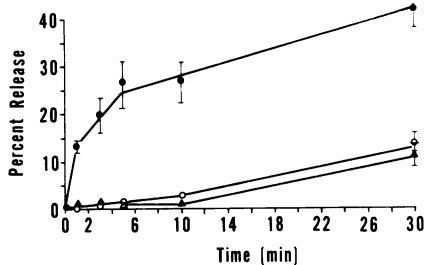


FIGURE 1 Time course of washed platelet release reaction induced by urate crystals. Urate crystals (0.8 mg/ml) were added to platelet suspensions. Incubations were terminated by rapid chilling followed by centrifugation. Percent release of serotonin (●—●), β -glucuronidase (○—○), and LDH (▲—▲) were determined. Values in this and other figures are mean \pm SEM. Where no bars appear, the SE is within the point.

TABLE I
Comparison of the Lytic Effect of Urate Crystals and Thrombin

	Percent release at 30 min incubation	
	Urate crystals	Thrombin
Serotonin (percent release)	42 ± 1.8	86 ± 0.9
LDH (percent loss)	16 ± 2.3	0.2 ± 0.4

800 μ l of [3 H]serotonin-labeled platelets in TBS pH 7.4 were incubated with 5 NIH units/ml crude bovine thrombin (Parke, Davis & Co.) or 0.8 mg/ml urate crystals. Percent release of serotonin and LDH were determined as noted in methods. Mean \pm SEM of triplicates.

modify platelet response to surfaces (16) and are known to affect the surface properties of urate crystals, these experiments were done in TBS, pH 7.4, but similar data was obtained using albumin density gradient-prepared cells. The dense body constituent (31), serotonin, was released within the first 10 min of incubation; in contrast, β -glucuronidase and cytoplasmic LDH were not detectably released during the first 10 min. ADP and ATP were released with the same time course

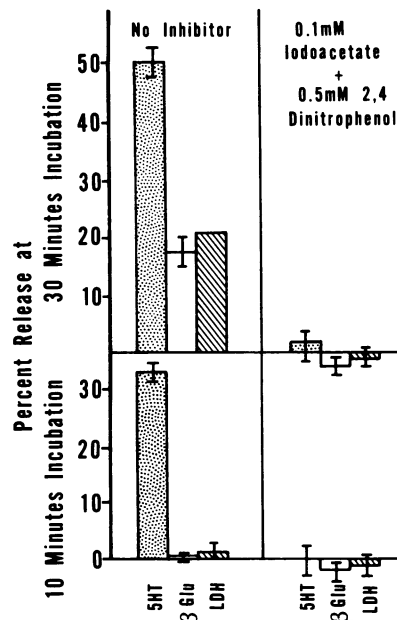


FIGURE 2 Effect of metabolic inhibitors on urate crystal-induced washed platelet release reaction. Washed platelet suspensions were incubated at 37°C for 10 min with either buffer (left panels) or a combination of 0.5 mM 2,4-DNP and 0.1 mM iodoacetate (right panels). Percent release of platelet constituents 10 (lower panels) and 30 (upper panels) min after addition of 1 mg/ml urate crystals was determined. 5HT = serotonin, β -glu = β -glucuronidase, LDH = lactic dehydrogenase.

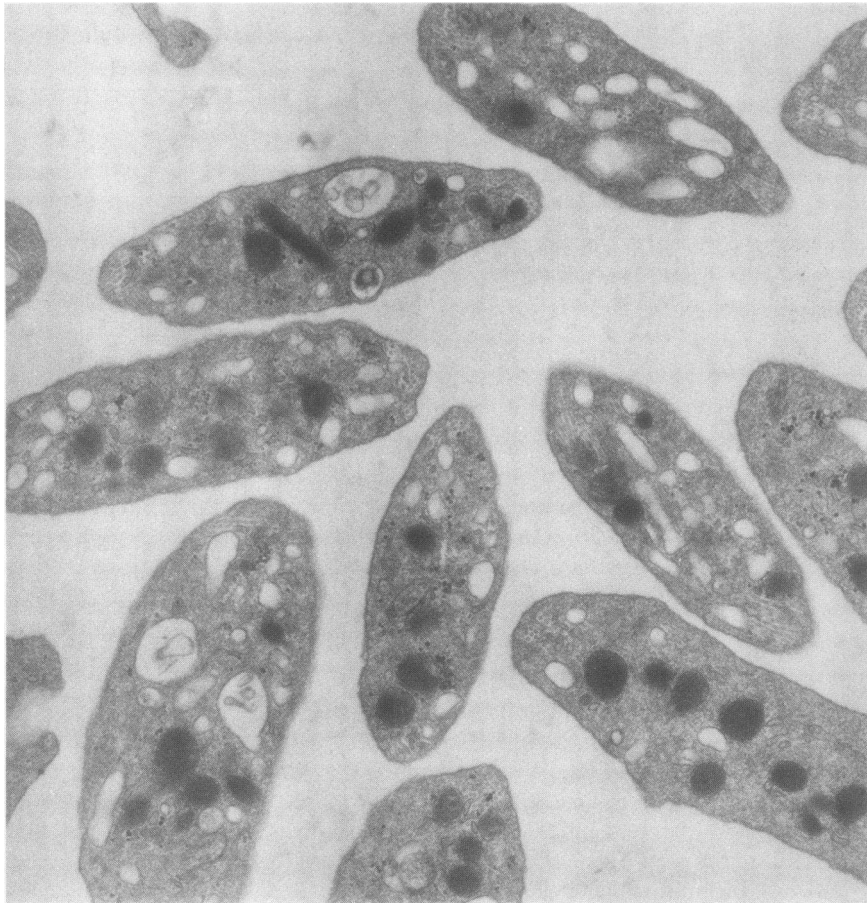


FIGURE 3 Albumin density gradient-prepared washed human platelets. $\times 56,000$.

as serotonin (not shown). This indicates that urate crystal-induced release of serotonin reflects release of other dense body constituents. In all control samples, less than 10% of total LDH was found in the supernatant (spontaneous lysis) after 30 min of incubation. At the crystal concentrations employed here, enzyme adsorption to crystals was undetectable but may have subtly contributed to the initial lag in appearance of β -glucuronidase and LDH in the supernatant.² From 10 to 30 min there was gradual release of all platelet constituents measured, suggesting a second phase of the reaction between platelets and crystals involving loss in platelet membrane integrity. Urate crystals were more active in lysing platelets than thrombin in that 5 times the thrombin required for maximal serotonin release at 30 min was unassociated with any loss of cytoplasmic LDH (Table I), while urate crystals induced substantial LDH loss at doses producing much less secretion of serotonin.

Effect of metabolic inhibitors. A combination of 0.1 mM iodoacetate and 0.5 mM 2,4-DNP, which blocks both anaerobic glycolysis and oxidative phosphoryla-

tion, inhibited both early and late components of serotonin release (Fig. 2). LDH and β -glucuronidase loss were inhibited as well. This suggests that urate crystal-induced lysis is initially an active process.

Effect of white cell contamination. PMNs alone in concentrations many times that present in our platelet suspensions released only a small fraction of the enzyme activity released by platelets. For example, incubation of a platelet suspension containing 8.5×10^7 platelets and 1.5×10^8 leukocytes/ml with 0.8 mg/ml urate crystals released 97.4 ± 14.7 Wroblewski units of LDH and 0.88 ± 0.11 pmol phenolphthalein per hour of β -glucuronidase activity. In contrast, 10^6 PMNs/ml released 8 ± 0.1 Wroblewski units of LDH and 0.02 ± 0.02 pmol phenolphthalein per hour of β -glucuronidase activity. Thus, the leukocytes in the platelet suspension could account for 0.01% of released LDH and 0.003% of released β -glucuronidase activities.

This indicates that in our platelet suspensions released enzymes were primarily of platelet origin. Deliberate PMN contamination up to 10^6 /ml was not associated with alterations in release of platelet constit-

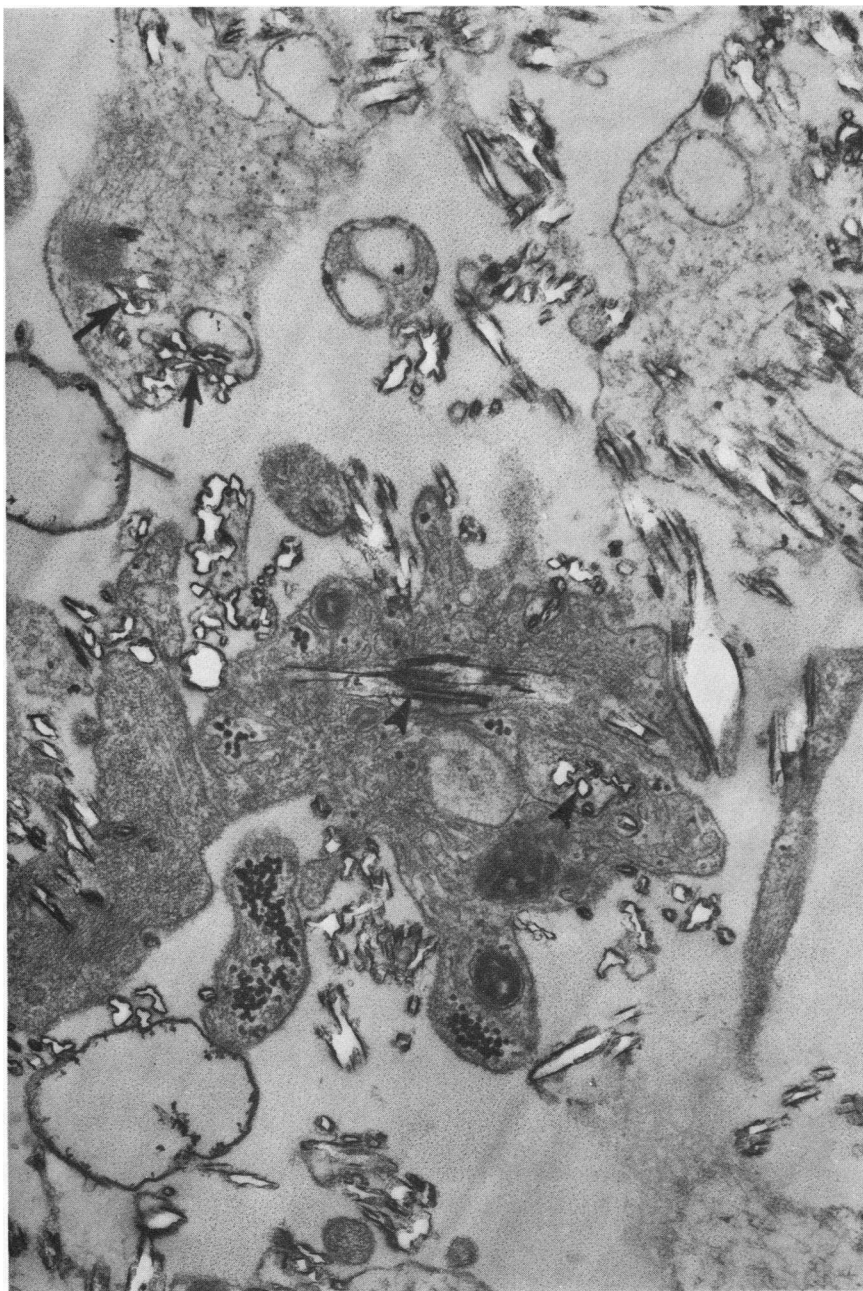


FIGURE 4 Effect of urate crystals on washed human platelets. Platelets prepared as above were incubated 37°C for 30 min with 2 mg/ml urate crystals. Black arrows: crystal artifacts in the cytoplasm of lysed cells. Arrowheads: crystal artifacts in membrane-lined compartment apparently in platelet cytoplasm. $\times 56,000$.

uents by urate crystals. In order to assess morphologic changes corresponding to the biochemical events of urate crystal-platelet interaction, ultrastructural studies were performed.

Morphologic studies. The albumin density gradient-prepared platelets (Fig. 3) were discoid, had intact membranes, contained storage granules, and had an

undilated open canalicular system. Urate crystals alone appeared either as holes in the plastic or as needle-shaped structures with electron-dense borders and less dense cores. Both types of artifact (Figs. 4 and 5) were seen in platelets incubated with urate crystals. The crystal-treated samples showed bizarre shape changes and degranulation. In some cells with intact cell mem-

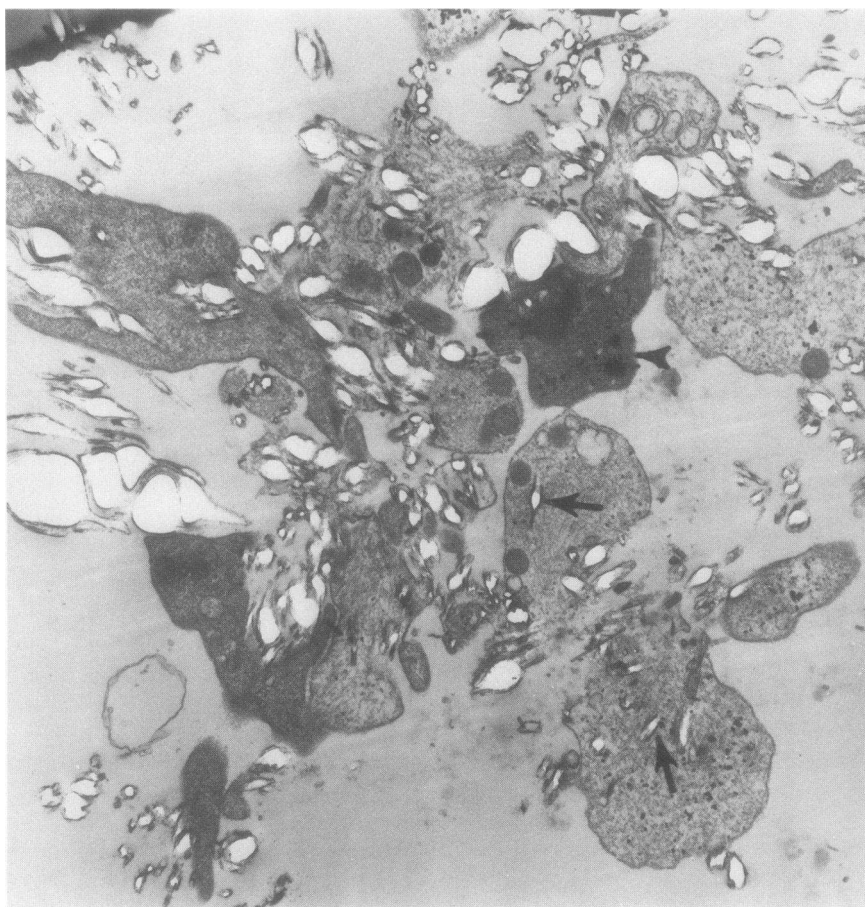


FIGURE 5 Effect of urate crystals on washed platelets. Treatment as described in legend to Fig. 4. Arrows: crystal artifact free in cytoplasm of lysed platelet. Arrowheads: platelet with intact cell membrane containing only one questionable crystal artifact. $\times 47,000$.

branes, crystal artifacts (Fig. 4) were noted inside membrane-lined compartments. In other cells (Figs. 4 and 5) there were huge gaps in the cell membrane and loss of cytoplasmic structure indicating cell lysis; in these cells, crystal artifacts (Figs. 4 and 5) were noted free in the cytoplasm. Platelets prepared in TBS also showed apparent crystal uptake and cell lysis, but the presence of shape change in buffer controls made assessment of this parameter impossible in TBS.

Studies in platelet-rich plasma

Because urate crystals activated washed platelets, their effects in platelet-rich plasma were examined.

Effect of crystals in PRP. Monosodium urate crystals induced release of serotonin in citrated PRP with peak release occurring at 10 min incubation. This property was shown by unheated crystals as well as by crystals heated to 200°C for 2 h. Different PRP preparations released from 5 to 45% of serotonin in response to 0.8 mg/ml urate crystals, indicating variability in indi-

vidual donors' platelet response to urate crystals in PRP.

Evidence that crystal-induced serotonin release in PRP is active. Since urate crystals are themselves membranolytic (32), it is possible that the serotonin release was due to a passive loss in platelet membrane integrity. As shown in Fig. 6, urate crystal-induced serotonin release in PRP was inhibitable by iodoacetate plus dinitrophenol or by EDTA, suggesting that it is an active process. Proteases generated subsequent to Hageman factor activation in plasma (33), such as thrombin, plasmin (34), and Factor Xa (35), have been reported to induce the platelet release reaction. Since crystals activate Hageman factor (36), the possibility that such interaction was the cause of crystal-induced serotonin release in PRP was explored.

Effect of Hageman factor activation in PRP. Buffer controls produced a recalcified clotting time of 220.7 ± 6.6 s (SE) and unheated urate crystals at 2 mg/ml 184.5 ± 14.7 s. Ellagic acid, an activator of Hageman factor (37), at a final concentration of $20 \mu\text{mol/liter}$ pro-

duced a clotting time of 101 ± 0.1 s; ellagic acid in this concentration did not induce serotonin release in PRP. This indicates that crystal-induced release is not solely due to Hageman factor activation. We next examined the question of urate crystal-induced platelet lysis in PRP.

Evidence of urate crystal-induced platelet lysis in PRP. In PRP, high plasma LDH precluded detection of minute quantities of platelet LDH loss. With platelets double labeled with ^{51}Cr and $[^{14}\text{C}]$ serotonin suspended in plasma, urate crystals provoked rapid serotonin release and later ^{51}Cr loss (Table II). This indicates that in PRP as in washed platelets, urate crystals provoke both secretion and lysis. It should be noted that urate crystals were tenfold less active in inducing lysis in PRP as in suspensions of washed platelets.

DISCUSSION

The time course of release of serotonin, ATP, and ADP induced by urate crystals in suspensions of washed platelets suggests a two-phase phenomenon: a rapid selective release of dense body constituents, and a slower loss of these constituents associated with loss of lysosomal and cytoplasmic enzymes.

Secretory phase. During the first 10 min incubation, serotonin, ATP, and ADP were selectively released without evident loss of LDH or β -glucuronidase. Inhibition of this phase of release with metabolic inhibitors indicates active platelet participation. Release of dense body constituents at 10 min incubation is then selective and active, i.e., is secretion.

Lytic phase. Serotonin release from 10 to 30 min

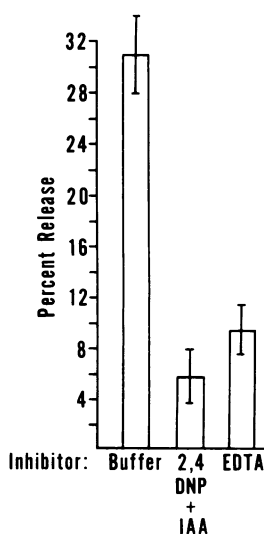


FIGURE 6 Effect of inhibitors on urate crystal-induced serotonin release in PRP. Buffer, 0.5 mM DNP + 0.1 mM iodoacetate (IAA), and 7.6 mM EDTA were preincubated with PRP 10 min before addition of 0.4 mg/ml urate crystals. Percent serotonin release was determined after 10 min incubation.

TABLE II
Urate Crystal-Induced Secretion and Lysis in PRP

	Urate crystals 2.5 mg/ml (time of incubation)		Urate crystals 10 mg/ml (time of incubation)	
	5 min	30 min	5 min	30 min
	% release			
$[^{14}\text{C}]$ serotonin	12.1 ± 0.4	17.4 ± 1.6	79.7 ± 4.2	97 ± 0.8
^{51}Cr	0.4 ± 0.2	3.7 ± 0.2	3.1 ± 0.4	20.7 ± 0.8

800 μl of 0.38% citrated plasma containing 2×10^8 $[^{14}\text{C}]$ serotonin- and ^{51}Cr -labeled platelets were incubated for the indicated times with 200 μl urate crystal suspension to yield the final concentrations shown. Reactions were stopped and percent release determined as shown in Methods. Mean \pm SEM of triplicates.

occurred in association with loss of LDH, indicating an increase in platelet membrane permeability. In ultrastructural studies, many of the platelets showed loss of cytoplasmic constituents and discontinuities in the cell membrane, suggesting that the slow loss of platelet constituents occurs by crystal-induced cell lysis. It is noteworthy that urate crystals induced 16% LDH loss in association with 42% serotonin release. In contrast, Kinlough-Rathbone et al. (38) reported that 5 times the dose of thrombin inducing maximal serotonin release (81.3%) from human platelets was associated with loss of only 1.6% of LDH. Similar data were obtained in our preparations. Thus, the lytic activity of the urate crystal for human platelets is greater than that of thrombin.

Henson (39) has demonstrated that neutrophils enhance rabbit platelet serotonin release induced by aggregated IgG or immune complexes, but that this phenomenon does not occur with human platelets and neutrophils (40). In our studies, increasing neutrophil contamination of up to 10^6 per ml did not alter the release reaction induced by urate crystals.

In vivo platelets are suspended in plasma. Thus, the demonstration that urate crystals induce active serotonin release in PRP adds weight to the possibility of crystal-induced platelet activation in vivo. The failure of ellagic acid, an activator of Hageman factor (37), to release serotonin in PRP shows that Hageman factor activation per se is not sufficient to account for urate crystal-induced serotonin release in PRP.

In PRP approximately 10 times as many urate crystals were required to initiate platelet lysis as in suspensions of washed platelets. Thus, platelets may be added to red cells (32) and neutrophils³ as cells in which plasma

³ Kozin, F., J. L. Skosey, M. Ginsberg, D. Chow, and J. May. Adsorption of proteins to monosodium urate crystals: crystal induced release of polymorphonuclear enzymes. Manuscript submitted for publication.

proteins inhibit urate crystal-induced lysis. This underscores the need for careful control of buffer protein content in the analysis of urate crystal-cell interactions.

Urate crystal deposition is not limited to articular structures in gouty patients; for example, crystals occur in subcutaneous tophi and heart valves (41) and have been reported in blood vessels (42). The role of platelet activation in intra- and extra-articular gouty inflammation would now seem to require further study. Similarly, urate crystals in blood vessels might lead to local platelet activation with endothelial damage (43) and possible mural thrombosis. Such a sequence of events could play a role in the increased platelet turnover (44) and risk of vascular disease in gouty patients (45).

ACKNOWLEDGMENTS

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