Enhancement of crystal induced neutrophil responses by opsonisation of calcium pyrophosphate dihydrate crystals

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Abstract

Objectives—Little is known about the effect on crystal induced neutrophil responses of the opsonisation of calcium pyrophosphate dihydrate (CPPD) (triclinic) crystals with components of serum and plasma. The purpose of this study was to determine the effects of precoating CPPD crystals with plasma, serum, complement depleted serum, and IgG on a full range of crystal induced neutrophil responses (calcium mobilisation, chemiluminescence, superoxide anion production, non-cytolytic lysosomal enzyme release, and leukotriene synthesis).

Methods—Crystals were precoated with IgG, serum, plasma, or complement depleted serum (heated at 56°C), incubated with neutrophils and the responses monitored with time. Measurement of the extent of neutrophil association with crystals was based on monitoring the decrease in fluorescence intensity of supernatants when crystals and diphenyl-hexatriene labelled neutrophils were allowed to settle under gravity.

Results—Precoating CPPD crystals with IgG, plasma, and serum significantly enhanced chemiluminescence, superoxide anion generation, increases in cytosolic free calcium levels, and non-cytolytic lysosomal enzyme release by neutrophils compared with uncoated CPPD crystals. The enhancement of neutrophil responses by crystals coated with complement depleted serum was less pronounced. The increased neutrophil responses induced by CPPD crystals coated with IgG might have been due to the observed increase in the association of IgG coated crystals with neutrophils.

Conclusions—These data show that there is a marked potentiation of all neutrophil responses to IgG, plasma, and serum coated CPPD crystals. It is suggested that the adsorption of synovial fluid proteins, including IgG and C3b, to CPPD crystals *in vivo*, results in the opsonised crystals becoming a potent neutrophil stimulant and inflammatory agent.

(*Ann Rheum Dis* 1993; 52: 599–607)

Calcium pyrophosphate dihydrate (CPPD) crystals in the synovial fluid of joints produce the inflammatory reaction of acute pseudo-

gout. This inflammation is now established as being derived primarily from the interaction of polymorphonuclear leucocytes or neutrophils with CPPD crystals. Previous *in vitro* studies have shown that neutrophils stimulated by synthetic CPPD crystals are slower to reach maximum response and produce less extensive neutrophil responses than neutrophils stimulated by synthetic monosodium urate monohydrate (MSUM) crystals.^{1–5}

Crystals of CPPD and MSUM have been shown to adsorb protein components from plasma and synovial fluid (an ultrafiltrate of plasma),^{6–9} and these adsorbed proteins are believed to exert a modulatory effect on the interaction of crystals and neutrophils. Indeed, crystals that have been precoated with synovial fluid or plasma components before incubation with neutrophils *in vitro* more closely model *in vivo* crystal-neutrophil interactions. Crystals of CPPD have been shown to adsorb small amounts of IgG, and CPPD crystals precoated with IgG alone produced no significant effects on neutrophil superoxide anion production compared with uncoated crystals.² Crystals of MSUM, on the other hand, adsorbed greater amounts of IgG leading to a significantly enhanced ability of the IgG coated crystals to stimulate neutrophils, with increased superoxide anion production and lysosomal enzyme release.^{2 10–12} The effects on crystal induced neutrophil responses of precoating crystals with plasma or serum have been studied extensively for MSUM but not for CPPD. Terkeltaub and coworkers showed that precoating MSUM with plasma inhibited chemiluminescence, superoxide generation, cytolysis, and phagocytosis,^{13 14} whereas Abramson *et al*¹¹ reported that MSUM induced neutrophil superoxide generation was inhibited by precoating the crystals with serum but not plasma. Kozin *et al*¹⁰ showed an inhibition of cytolysis but an enhancement of lysosomal enzyme release when MSUM crystals were coated with serum.

In this work we have investigated a full range of neutrophil responses to CPPD crystals, the effect of opsonisation with IgG, and the effect of precoating CPPD crystals with plasma or serum. Although published reports suggest that CPPD crystals are relatively weak stimulators of neutrophil responses compared with MSUM,^{1 2 4} we have shown that CPPD crystals are strong activators of neutrophil responses. Also, contrary to the effects of plasma opsonisation of MSUM, which has been shown in general to inhibit neutrophil

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Accepted for publication
20 April 1993

responses, we have shown that opsonisation of CPPD crystals with plasma or serum significantly enhanced CPPD induced neutrophil responses.

Materials and methods

BUFFER

Hanks's buffered salt solution (HBSS), pH 7.4, was used throughout.

PREPARATION AND CHARACTERISATION OF CRYSTALS

Crystals of CPPD (triclinic) were prepared and were characterised using x ray diffraction and differential scanning calorimetry as previously described,¹⁵ and were used unheated in all studies. x Ray diffraction patterns showed all d spacings characteristic of triclinic CPPD crystals and there was no evidence of any additional peaks due to other crystalline phases such as monoclinic CPPD. Two endothermic peaks on the differential scanning calorimetry thermograms corresponding to the loss of two moles of water from the solid indicated the presence of the dihydrate.¹⁵ The size distribution of the crystals was approximately 33% less than 10 μm , 58% between 10 and 20 μm , and 9% greater than 20 μm . Crystals of CPPD were washed in HBSS using the same procedure as for precoating crystals with proteins, and were tested for the presence of pyrogens using the Limulus amebocyte assay (Sigma kit No 210-A1-E-Toxate). The CPPD samples were free of pyrogens.

NEUTROPHIL PREPARATION

Neutrophils were prepared by dextran sedimentation and Ficoll Paque (Pharmacia) density centrifugation. The pellets were washed free of erythrocytes by 20 seconds of hypotonic shock using distilled water followed by the restoration of tonicity with 0.6 M NaCl. Neutrophils were resuspended in HBSS, stored on ice, and used for experiments within three hours. Neutrophils always represented greater than 90% of the suspension, and cell viability, as estimated by trypan blue exclusion, was greater than 95%.

INCORPORATION OF FURA 2AM OR DIPHENYLHEXATRIENE INTO NEUTROPHILS

Cells (5×10^6 cells/ml) in either 1 μM fura 2AM (Sigma Chemical) or 1 μM diphenylhexatriene (Sigma) in HBSS were incubated at room temperature for 30 minutes, centrifuged at 400 g for six minutes, washed, and resuspended in HBSS.

CYTOSOLIC CALCIUM DETERMINATIONS

All determinations were made at 37°C. Fluorescence emission from 1.5 ml fura 2 loaded cells in the presence or absence of crystals was recorded continuously by excitation at 340 nm and emission at 500 nm. Increases in the values of the fluorescence

intensities (F) from fura 2 loaded neutrophils due to light scattering effects from crystals were corrected by subtraction of the scatter intensity (F_{scat}). Cytosolic free calcium concentrations $[\text{Ca}^{2+}]_i$ were determined using the following equation

$$[\text{Ca}^{2+}]_i = K_d \frac{(F - F_{\text{min}})}{(F_{\text{max}} - F)}$$

where F_{max} and F_{min} are the (scatter corrected) fluorescence intensities at maximum (0.02% Triton X-100) and minimum (50 mM EGTA) calcium concentrations and K_d is the dissociation of fura 2 with Ca^{2+} and is equal to 224 nmol/l.¹⁶ To measure light scattering intensities produced by adding crystals to suspensions of fura 2 loaded neutrophils, neutrophils were treated with 0.02% Triton X-100 to saturate fura 2 with calcium. The fluorescence intensities (at 340 nm excitation) were monitored. Crystals were added in incremental amounts and any subsequent increases in fluorescent intensity were treated as scatter intensities (F_{scat}).

DETERMINATION OF CRYSTAL-NEUTROPHIL ASSOCIATION

Diphenylhexatriene labelled neutrophils (1.5 ml) at 5×10^6 cells/ml were added to a fluorescence cuvette at 37°C and the emission spectrum recorded from 400 to 475 nm using 355 nm excitation and a Shimadzu RF 540 spectrofluorophotometer. Crystals of CPPD (75 mg) were added and stirred for 10 minutes at 37°C using a stir bar accessory (Hellma Canada). The stirrer was switched off and the contents of the cuvette allowed to sediment under gravity for two minutes. The emission spectra of the supernatants were recorded. The percentage neutrophil association with crystals was determined using the following equation

$$\text{Neutrophil association (\%)} = (1 - F_0/F_c) \times 100$$

where F_0 is the fluorescence emission intensity at 428 nm for diphenylhexatriene loaded neutrophils alone (crystals absent) and F_c is the fluorescence emission intensity at 428 nm of supernatants after gravity sedimentation of crystal-neutrophil suspensions.

CHEMILUMINESCENCE

Cells (5×10^6), 1 μM luminol (dissolved in dimethylsulphoxide) and 50 mg CPPD crystals were mixed and chemiluminescence monitored using an LKB Luminometer (Model 1250) at 37°C with shaking immediately before measurements. Control tubes contained cells and luminol (crystals absent).

SUPEROXIDE ANION GENERATION

Eppendorf tubes containing CPPD crystals (final concentration, 50 mg/ml), 1 mg/ml ferricytochrome c (horse heart type 3; Sigma), and cells (5×10^6 /ml) were incubated at 37°C by tumbling end over end at 100 rev/min. Identical tubes also containing 600 U/ml

superoxide dismutase (Sigma) were tumbled at 37°C. At given time intervals the tubes were removed, centrifuged at 10 000 *g* for 10 seconds and the supernatant removed for immediate spectrophotometric assay at 550 nm. The concentration of superoxide anion in the supernatant was calculated using an extinction coefficient of 0.0211 for ferricytochrome *c*.

NEUTROPHIL DEGRANULATION

Cells (5×10^6 /ml) and CPPD crystals (50 mg/ml) were incubated at 37°C by tumbling end over end at 100 rev/min in microfuge tubes and at given time intervals the tubes were removed, centrifuged in a microfuge for 10 seconds, and 0.4 ml supernatant frozen at -20°C for later assay. Lysozyme was measured by adjusting the absorbance of a 2.5 ml *Micrococcus lysodeikticus* (Sigma) suspension (0.1 mg/ml in 0.65 M potassium phosphate buffer, pH 6.2) to 0.7, adding 100 µl supernatant, and monitoring the decrease in absorbance with time. Lysozyme standards (chicken egg white; Sigma) in the 0–2000 U/ml range were prepared and a standard curve obtained. Myeloperoxidase activity was determined by monitoring the change in absorbance per minute (ΔA_{450}) of 0.89 ml 3.2 mM *o*-dianisidine (Sigma) solution in 0.1 M citrate buffer, pH 5.5, to which had been added 10 µl 10 mM hydrogen peroxide, 50 µl 1% Triton X-100, and 50 µl supernatant. Myeloperoxidase activity was calculated from the following relationship

$$\text{Dianisidine oxidation (nmol/min)} = 50 \Delta A_{450}$$

Supernatants from these degranulation experiments were also assayed for lactate dehydrogenase levels as previously described.³

LEUKOTRIENE GENERATION

Cells (3×10^7 /ml) and CPPD (100 mg/ml) were tumbled at 37°C as previously described for 30 minutes, centrifuged at 10 000 *g* for 10 seconds and the supernatants collected for leukotriene extraction.¹⁷ Briefly, 1.5 ml supernatant was added to conditioned C18 extraction cartridges (Baker) and leukotrienes eluted with 5 ml methanol, evaporated to dryness under nitrogen and reconstituted in 200 µl methanol. Leukotrienes were measured by reversed phase high performance liquid chromatography (HPLC) using ultraviolet detection at 280 nm by the method of Steffenrud and Salari.¹⁸ The HPLC system consisted of a Beckman Model 110A pump, a Model 210 sample injection valve fitted with a 20 µl loop, a Beckman Model 160 ultraviolet detector, and a Shimadzu C-R3 integrator. The column was an ODS C18 silica column (Beckman) and the mobile phase was methanol/water, 7:3 v/v, containing 0.1% heptafluorobutyric acid adjusted to pH 3.0 with triethylamine at a flow rate of 1 ml/min. The internal standard, was added to the sample before extraction.

PRECOATING CRYSTALS WITH PROTEINS

Crystals were incubated by tumbling with 50% normal human serum, 50% plasma (freshly collected using heparin anticoagulant) or 6 mg/ml IgG (human; Sigma) at 37°C for 30 minutes. Zymosan (2 mg/ml) was also incubated with 50% serum. Following centrifugation at 10 000 *g* for 10 seconds, the crystal pellets were washed in HBSS and recentrifuged.

IgG BINDING TO CRYSTALS

Crystals of CPPD (6.25 mg) that had been precoated with plasma or IgG were boiled for 1.5 minutes in 100 µl 2% sodium dodecyl sulphate (SDS) containing 0.1 M dithiothreitol. The supernatant (20 µl) was analysed by polyacrylamide gel electrophoresis (PAGE) using a 7.5% gel in a Mini-protein 2 dual slab electrophoresis system (Bio Rad, Richmond, CA, USA). Bands were visualised by Coomassie blue staining. Crystals of CPPD (50 mg/ml) were tumbled at 37°C for 30 minutes with fluorescein isothiocyanate conjugated human IgG (6 mg/ml) (Sigma). After centrifugation at 10 000 *g* for 10 seconds, the crystal pellets were washed twice in excess HBSS and recentrifuged. The crystal pellets were then vortexed and boiled in 2% SDS containing 0.1 M dithiothreitol to release any bound fluorescein labelled IgG. The fluorescein labelled IgG was then measured using fluorescence spectroscopy at 480 nm excitation, 520 nm emission (Shimadzu spectrofluorometer).

ADSORPTION OF ENZYMES AND LEUKOTRIENES BY CPPD CRYSTALS

Supernatants from degranulation studies were collected, 50 µl retained for enzyme analysis, and the remaining portion incubated with uncoated or plasma coated CPPD crystals (50 mg/ml) at 37°C for 10 minutes. After centrifugation at 10 000 *g* for 10 seconds, supernatants were assayed for myeloperoxidase or lysozyme and the results compared with myeloperoxidase or lysozyme assay values from control (50 µl) samples. Leukotriene adsorption was carried out by incubating CPPD crystals (50 mg/ml) with a solution of 1 mg/ml each of leukotriene B₄ (LTB₄), the LTB₄ omega oxidised metabolite LTB₄-OH, and prostaglandin B₂ for 10 minutes at 37°C.

Results

Concentrations of neutrophils and crystals found to produce significant and reproducible levels of neutrophil activation and responses were 5×10^6 cells/ml and 50 mg/ml CPPD crystals. These concentrations were used in all studies except for leukotriene determinations where higher cell and crystal concentrations were needed to produce sufficient amounts of leukotrienes for measurement.

Figures 2, 4, 5, and 6 each show representative time course data for one experiment.

Table 1 shows a statistical treatment of pooled data from several time course experiments (between three and 12 experiments).

CRYSTAL-NEUTROPHIL ASSOCIATION

These experiments were adapted from previously published methods for measuring the binding or association of MSUM to platelets¹⁴ and erythrocytes.¹⁹ The studies were based on monitoring the decrease in fluorescence intensity of supernatants when diphenylhexatriene labelled neutrophils were mixed with crystals and allowed to settle under gravity. This process largely sedimented crystals and neutrophils associated with crystals but not 'free' neutrophils, which therefore remained in the supernatant. The 'free' neutrophils in the supernatant could be determined by reading the fluorescence intensity of the supernatant, this intensity being the total diphenylhexatriene emission from the neutrophils in the supernatant. A series of dilutions of a neutrophil suspension from 5×10^6 to 5×10^5 cells/ml showed a linear decrease in fluorescence intensity at 428 nm with a correlation coefficient of 0.9997.

This so called association of neutrophils and crystals could include neutrophils with crystals bound to the plasma membrane, or neutrophils with internalised or phagocytosed crystals, or both. Preliminary experiments to optimise the crystal concentration at a cell concentration of 5×10^6 cells/ml showed that the percent association of crystals with neutrophils was 10, 17, 27, and 30% at crystal concentrations of 4.5, 9, 18, and 27 mg/ml. At a crystal and cell concentration of 50 mg/ml and 5×10^6 cells/ml, the percentage association of crystals with neutrophils was 53 (11)%. Figure 1 shows the effect of protein precoating of CPPD on the percentage neutrophil association at 50 mg/ml CPPD crystals. Precoating CPPD crystals with plasma, serum, or serum heated at 56°C had no effect on the percentage neutrophil association. IgG coated CPPD crystals significantly increased the percentage neutrophil

association (78 (2)%) compared with uncoated CPPD crystals ($p < 0.01$).

CHEMILUMINESCENCE

The chemiluminescent response of neutrophils to uncoated CPPD crystals increased significantly as the crystal concentration was increased, the peak response at 10 mg/ml CPPD crystals being only approximately 20% of the peak response at 50 mg/ml. Crystal concentrations greater than 50 mg/ml produced decreased chemiluminescence, probably owing to interference of the high concentration of suspended material with light emission. The chemiluminescent responses of neutrophils stimulated by plasma, serum, and heated serum precoated CPPD crystals were faster and more extensive than neutrophils stimulated by 50 mg/ml uncoated CPPD crystals (fig 2). Similar results were obtained for IgG coated CPPD crystals. Maximum chemiluminescent response was reached in about three to five minutes for uncoated CPPD crystals and in about two to three minutes for plasma, serum, heated serum, and IgG coated CPPD.

Maximum neutrophil chemiluminescent responses to all protein coated CPPD crystal samples were significantly higher than maximum chemiluminescent responses to uncoated CPPD crystals ($p < 0.01$). Table 1 gives the increases in neutrophil response to protein coated relative to uncoated CPPD crystals. Crystals of CPPD coated with serum heated at 56°C to deplete complement induced a lower chemiluminescence response than serum coated CPPD crystals. Similar results were obtained with zymosan coated with serum or complement depleted serum.

CYTOSOLIC FREE CALCIUM, $[Ca^{2+}]_i$

The value of $[Ca^{2+}]_i$ increased above resting (control) neutrophil levels when neutrophils were stimulated by uncoated CPPD crystals, reaching a maximum value at four minutes. $[Ca^{2+}]_i$ at crystal concentrations of 5, 10, 20, and 30 mg/ml were approximately 60, 120, 150, and 200 nmoles respectively. $[Ca^{2+}]_i$ at 50 mg/ml was about 320 nmoles. At CPPD crystal concentrations greater than 50 mg/ml fluorescence emission values were decreased. Figure 3 and table 1 show that CPPD crystals coated with serum, complement depleted serum, plasma or IgG all induced greater increases in $[Ca^{2+}]_i$ above resting levels than uncoated CPPD. All $[Ca^{2+}]_i$ increases due to protein coated crystals were significantly higher than $[Ca^{2+}]_i$ increases due to uncoated crystals ($p < 0.01$).

SUPEROXIDE ANION GENERATION

Figure 4 shows the time course of superoxide dismutase inhibitable superoxide anion generation by neutrophils stimulated by plasma, serum, and IgG coated CPPD and uncoated CPPD crystals. All protein coated crystals resulted in a significant enhancement ($p < 0.01$)

Figure 1 Effect of precoating calcium pyrophosphate dihydrate (CPPD) crystals with plasma, IgG, serum, and complement depleted serum on diphenylhexatriene labelled neutrophil association with crystals at 37°C. Crystal concentration, 50 mg/ml; neutrophil concentration, 5×10^6 cells/ml.

Figure 2 Time course of luminol enhanced chemiluminescent response of neutrophils (5×10^6 cells/ml) stimulated by calcium pyrophosphate dihydrate (CPPD) crystals (50 mg/ml) at 37°C . (▲) Control cells; (△) uncoated CPPD crystals; (○) serum coated CPPD crystals; and (●) plasma coated CPPD crystals. Inset: (▲) uncoated CPPD; (●) complement depleted serum coated CPPD; and (○) serum coated CPPD.

Table 1 Percentage increases in neutrophil responses at 37°C to protein coated calcium pyrophosphate dihydrate (CPPD) crystals relative to uncoated CPPD crystal (CPPD, 50 mg/ml; neutrophils, 5×10^6 /ml)

Neutrophil response	Percentage increase in response to protein coated CPPD relative to uncoated CPPD*			
	Plasma coated	Serum coated	Coated with serum treated at 56°C	IgG coated
Chemiluminescence†	66 (29) (n=9)	61 (23) (n=9)	22 (12) (n=8)	61 (26) (n=10)
Calcium mobilisation†	82 (56) (n=12)	91 (42) (n=12)	69 (43) (n=10)	47 (24) (n=5)
Superoxide anion generated‡	123 (51) (n=4)	108 (45) (n=7)	53 (34) (n=4)	116 (58) (n=4)
Degranulation: lysozyme release§		55 (14) (n=4)	21 (5) (n=3)	91 (17) (n=4)

*Mean (SD) percentage increase in response = $100 [(R_p - R_c) - (R_{un} - R_c) / (R_{un} - R_c)]$ where R_p = neutrophil response to protein coated CPPD, R_c = control neutrophil response (CPPD absent) and R_{un} = neutrophil response to uncoated CPPD.

†Response measured at maximum.

‡Response measured at 10 minutes.

§Response measured at 17 minutes.

of superoxide generation by neutrophils compared with uncoated crystals (measured at 10 minutes). Table 1 gives the values of the percentage increases (at 10 minutes). To verify

these results obtained with the ferricytochrome *c* reduction assay, we used the nitro blue tetrazolium assay²⁰ to measure superoxide generation in a single time course experiment.

Figure 3 Changes in neutrophil (5×10^6 cells/ml) cytosolic free calcium concentrations after four minutes' incubation with calcium pyrophosphate dihydrate (CPPD) crystals (50 mg/ml) at 37°C . (A) Effect of serum or plasma coating of CPPD crystals. (B) Effect of serum or heat treated (complement depleted) serum coating of CPPD.

Figure 4 Time course of superoxide anion release from neutrophils (5×10^6 cells/ml) stimulated by calcium pyrophosphate dihydrate (CPPD) crystals (50 mg/ml) at 37°C . (○) Control cells; (●) uncoated CPPD crystals; (□) plasma coated CPPD crystals; (△) IgG coated CPPD crystals, and (▲) serum coated CPPD crystals.

A similar time course profile of superoxide generation to that in fig 4 was produced by uncoated CPPD crystals, and plasma coated CPPD crystals induced significantly greater levels of superoxide by neutrophils.

NEUTROPHIL DEGRANULATION

Figure 5 shows the time course of CPPD crystal induced neutrophil degranulation, as monitored by extracellular lysozyme concentration. Coating CPPD crystals with plasma, serum, complement depleted serum, or IgG produced significantly higher levels of lysozyme release than uncoated CPPD ($p < 0.01$) (fig 5 and table 1). Table 1 gives the values of the percentage increases of lysozyme release measured at 17 minutes due to protein coated crystals relative to uncoated crystals. Myeloperoxidase release from neutrophils may be measured by monitoring the rate of oxidation of dianisidine and hence the time course of CPPD induced myeloperoxidase release from

neutrophils is given in fig 6. In a single time course experiment serum and IgG coating of CPPD crystals produced a greater release of myeloperoxidase from neutrophils than uncoated CPPD.

Supernatants from degranulation experiments were assayed for lactate dehydrogenase release. Over the 40–50 min period of incubation of neutrophils and crystals in these studies, we found no significant release of lactate dehydrogenase from neutrophils over controls, indicating that myeloperoxidase and lysozyme release was not simply a result of neutrophil cytolysis.

LEUKOTRIENE RELEASE

Significant amounts of the LTB_4 omega oxidised metabolite, $\text{LTB}_4\text{-OH}$, were synthesised by neutrophils in response to stimulation by CPPD crystals for 30 minutes (table 2). Neutrophils were also incubated

Figure 5 Time course of neutrophil degranulation as measured by lysozyme release from neutrophils (5×10^6 cells/ml) stimulated by calcium pyrophosphate dihydrate (CPPD) crystals (50 mg/ml) at 37°C . (○) Control cells; (●) uncoated CPPD crystals; (▲) serum coated CPPD crystals, and (□) plasma coated CPPD crystals.

Figure 6 Time course of neutrophil degranulation as measured by myeloperoxidase release from neutrophils (5×10^6 cells/ml) (myeloperoxidase activity expressed as the rate of oxidation of dianisidine in nmol/min) stimulated by calcium pyrophosphate dihydrate (CPPD) crystals (50 mg/ml) at 37°C. (○) Control cells; (●) uncoated CPPD crystals; (△) IgG coated CPPD crystals; and (▲) serum coated CPPD crystals.

Table 2 Recovery of leukotriene B_4 omega oxidised metabolite (LTB_4 -OH) from neutrophil incubations (3×10^7 cells/ml) with uncoated or plasma coated calcium pyrophosphate dihydrate (CPPD) (100 mg/ml) at 37°C for 30 minutes

Experiment number	LTB ₄ -OH recovery (pmol/ 10^7 cells)	
	Uncoated CPPD	Plasma coated CPPD
1	110 (20)	90 (6)
2	450 (78)	570 (382)
3	188 (53)	428 (128)

with ionomycin, a potent activator of the 5-lipoxygenase pathway, and the amount of LTB_4 -OH recovered was 16 nmol/ 10^7 cells. In two of three experiments there was no significant difference between LTB_4 -OH released from neutrophils stimulated by uncoated versus plasma coated CPPD crystals. Experiment 3 showed that plasma coated CPPD crystals induced significantly increased release of LTB_4 -OH from neutrophils compared with uncoated CPPD crystals.

IgG BINDING TO CRYSTALS

Sodium dodecylsulphate polyacrylamide gel electrophoresis of an IgG standard (4 µg total protein was applied to the lane) gave characteristic bands for the heavy and light chains of the dissociated protein. Sodium dodecylsulphate eluates from IgG precoated CPPD crystals gave identical band patterns to the IgG standard. Eluates from plasma precoated CPPD crystals also showed bands which were weaker than those of eluates from IgG precoated crystals at the same molecular weight as the IgG bands. Measurement of binding of FITC IgG conjugate to CPPD crystals showed that at the concentration of IgG used for opsonisation (6 mg/ml), CPPD crystals (50 mg/ml) bound 47 (8) µg (four samples) of IgG or 0.94 µg IgG/mg CPPD crystals.

ADSORPTIVE CAPACITY OF CPPD CRYSTALS

We found that neither plasma precoated nor uncoated CPPD crystals adsorbed significant amounts of myeloperoxidase or lysozyme. In addition, there was no significant adsorption of leukotrienes or prostaglandin B_2 by either uncoated CPPD crystals or plasma precoated CPPD crystals. By way of comparison, uncoated MSUM at 5 mg/ml adsorbed negligible amounts of lysozyme but 85 (10)% (three samples) of the myeloperoxidase from identical concentrations of enzymes used in CPPD crystal adsorption experiments.

Discussion

Previous in vitro studies have shown that the interaction of uncoated triclinic CPPD crystals with neutrophils results in phagocytosis,¹ increases in $[Ca^{2+}]_i$,⁵ and the generation of the superoxide anion.²⁻⁵ Neutrophil cytolysis required long incubation times.³ Monoclinic crystals of CPPD induced leukotriene synthesis, particularly production of LTB_4 and its omega oxidised metabolites.²¹

In this work, under conditions of high levels of crystal-neutrophil association, uncoated CPPD crystals were a potent activator of neutrophils resulting in calcium mobilisation, chemiluminescence, superoxide anion production, non-cytolytic lysosomal enzyme release, and leukotriene synthesis.

Previous studies comparing neutrophil activation by CPPD and MSUM crystals at equivalent concentrations (generally between 3 and 8 mg/ml) have shown that CPPD crystals always produced significantly lower neutrophil responses than MSUM crystals.^{2-4,5} Our results show that much greater CPPD crystal concentrations (50 mg/ml) were required to produce high levels of neutrophil association and activation. The differences in responses to CPPD and MSUM crystals may be explained at least in part by the difference in surface area of CPPD and MSUM crystals. The long needle shaped habit of the MSUM crystals gives a high surface area to volume ratio compared with the prismatic shape of the CPPD crystal, and specific surface areas of our MSUM samples are generally five to tenfold higher than specific surface areas of our synthetic CPPD triclinic samples.³ Hence for a given concentration of MSUM and CPPD, this should lead to a greater surface area of MSUM crystal available for contact with the neutrophil plasma membrane than CPPD crystals.

All neutrophil responses to uncoated and protein coated CPPD crystals have been monitored in the absence of cytochalasin B, a strong inhibitor of phagocytosis commonly used in crystal-neutrophil interaction studies. We feel that the exclusion of cytochalasin B better represents the in vivo CPPD-neutrophil interaction. Cytochalasin B has been reported to inhibit MSUM induced chemiluminescence,¹⁴ oxygen uptake,²⁰ and degranulation (α mannosidase release).¹⁴ Cytochalasin B has also caused enhancement and inhibition of the neutrophil superoxide

anion release induced by a number of particulate and soluble stimuli, which may be related to the nature of the opsonin.^{22 23}

The adsorption of IgG to CPPD crystals after precoating the crystals with either IgG or plasma was confirmed. Precoating CPPD crystals with IgG, plasma, or serum enhanced all neutrophil responses with the exception of leukotriene generation, where only one of three experiments showed enhanced LTB₄-OH recovery after stimulation of neutrophils with plasma coated CPPD crystals. IgG coating of CPPD crystals caused an increase in the association of crystals with neutrophils from 53% for uncoated CPPD crystals to 78%, but plasma and serum coating of CPPD crystals had no effect on the percentage association of crystal with cells. It is possible that the enhanced neutrophil responses induced by IgG coated CPPD crystals might have been due entirely or in part to the increase in association of IgG coated crystals with neutrophils.

IgG and the complement component C3b are well characterised opsonising proteins which activate a number of plasma membrane receptors leading to activation of the neutrophil. Although incubation of CPPD crystals with plasma might have resulted in the adsorption of a number of different protein components from plasma, it appears that there was no masking or interference of the opsonising effects of proteins such as IgG or C3b, or both, by other adsorbed protein species. The effects of precoating CPPD crystals with plasma or serum on crystal induced neutrophil responses were compared owing to the possible adsorption of fibrinogen onto CPPD crystals (MSUM has been shown to adsorb fibrinogen⁸). There was no significant difference between the abilities of either plasma precoated CPPD or serum precoated CPPD crystals to enhance neutrophil responses. It is likely that even if additional proteins are adsorbed onto the crystals from plasma, they do not mask the potentiating effects of IgG or C3b adsorbed on CPPD.

Heating serum at 56°C for 30 minutes is a commonly used method to deplete serum of complement.¹¹ Heating serum at 63°C causes aggregation of IgG,²⁴ however, and in precoating crystals with 56°C heat treated serum there may have been a decreased adsorption of IgG onto the crystal surface as well as a lack of adsorbed complement species. This might explain the reduced (but not abolished) ability of crystals coated with heat treated serum to enhance neutrophil responses compared with crystals coated with normal serum.

There are two broad categories of agents that are capable of activating neutrophils via receptor and non-receptor mediated pathways. Receptor mediated activation of neutrophils by opsonised particles, such as yeast or zymosan, occurs through specific receptors for C3b or the Fc domain of IgG. The signal transduction pathways for Fc receptor and C3b receptor mediated neutrophil responses are the subject of many literature reports.²⁵⁻²⁸ Similarly, the activation pathways for neutrophils stimulated by inflammatory microcrystals, such as MSUM and CPPD crystals, which have no

apparent specific receptor on the neutrophil membrane are currently under investigation.^{5 29-31} It may well be that the pathways leading to crystal induced neutrophil activation may be different for uncoated crystals and for serum/plasma/IgG opsonised crystals. Studies of the neutrophil activation pathways for crystals precoated with plasma, serum, and IgG are in progress.

It has been hypothesised by a number of workers that synovial fluid proteins adsorbed onto crystals are important factors regulating crystal induced inflammation. Synovial fluid is an ultrafiltrate of plasma and proteins are present in low concentrations in normal synovial fluid.^{32 33} In inflammatory states, however, the protein concentration becomes increased with increased levels of immunoglobulins, lipoproteins, fibrinogen, and variable levels of complement components.^{32 33} This is the first report showing a marked potentiation of all neutrophil responses to IgG, plasma, and serum coated CPPD crystals. We suggest that the adsorption of synovial fluid proteins, including IgG and C3b, to CPPD crystals in vivo results in the opsonised CPPD crystals becoming a potent neutrophil stimulant and inflammatory agent.

The study was supported by a grant from the Medical Research Council of Canada. The authors thank the Canadian Red Cross, Vancouver for supplying the human blood and the medical laboratory of the University Hospital (UBC site) University of British Columbia for the analysis of the cell suspensions.

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