A biochemical study of hydroxyapatite crystal induced enzyme release from neutrophils

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SUMMARY Addition of hydroxyapatite (HyAp) microcrystals to human neutrophils results in exocytosis of specific granules, measured as lysozyme release, and plasma membrane damage, evident from lactate dehydrogenase (LDH) release. The strong hydrogen acceptor polyvinylpyridine-*N*-oxide has no effect on enzyme release, but polyanions and negatively charged proteins such as albumin strongly inhibit HyAp induced enzyme release. HyAp crystals cause only slightly less membrane damage in neutrophil cytoplasts than in intact neutrophils. Removal of sialic acid from the cells did not affect HyAp induced enzyme release. Glucose, trapped in negatively charged liposomes, is released by HyAp crystals, whereas the crystals do not release glucose from positively charged liposomes. The results indicate that positive charges located on the HyAp crystals are of predominant importance for the effect of the crystals, and that the lipid part of the membrane might play an important part in the interaction.

Key words: positive charges.

Microcrystals of hydroxyapatite (HyAp) have been observed in association with a number of pathogenic conditions. HyAp crystals have been found in patients with several types of arthritis, such as osteoarthritis, bursitis, periarthritis, erosive arthritis, and in the Milwaukee shoulder syndrome.¹⁻¹⁰ When HyAp crystals are injected in vivo at appropriate places a severe inflammation results.^{11, 12}

The precise mechanisms by which HyAp crystals cause inflammation are largely unknown. In recent years, however, a number of studies have been performed to clarify this. HyAp crystals are able to interact with several cell types, eventually resulting in the release of inflammation promoting constituents.¹²⁻¹⁵

Neutrophils are associated with a wide range of inflammatory conditions, owing to their ability to release tissue degrading enzymes and to produce toxic oxygen metabolites upon activation.¹⁶⁻¹⁸ Intrapleural injection of HyAp in the rat causes an inflammatory reaction consisting of exudate and a mixed leucocyte population of neutrophils and mononuclear cells.¹⁹

The ability of the neutrophil to release tissue degrading enzymes and inflammation promoting constituents upon activation, and the presence of that cell type in the vicinity of HyAp depositions, suggests that the neutrophil may serve as a possible mediator in HyAp crystal associated inflammations.

For this reason a biochemical study was undertaken to observe the interaction between HyAp microcrystals and neutrophils and thus determine the ability of the crystals to release cellular constituents from neutrophils and make an approach towards establishing the mechanism of this release.

We stated in a preliminary report that HyAp crystals may cause cell damage in erythrocytes and rabbit peritoneal neutrophils, that phagocytosis may be involved in crystal induced cell damage in rabbit neutrophils, and that positive charges might play a part in cell-crystal interaction.¹⁴ Using human neutrophils we have elaborated the experimental basis of these statements in an attempt to localise the target of HyAp crystals in the cell.

Materials and methods

NEUTROPHILS

Human neutrophils were isolated from heparinised blood by dextran sedimentation, followed by lysis of the remaining erythrocytes and separation of the

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white blood cells by the Ficoll-Isopaque technique, according to the method described by Böyum.²⁰ The medium used consisted of 140 mM NaCl, 5 mM KCl, 10 mM glucose, and 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid) pH 7.3. Before the experiment 1 mM Ca⁺⁺ and 1 mM Mg⁺⁺ were added to the medium. Neutrophils (3×10^6 /ml) were incubated for 30 min at 37°C. Then 100 µl 0.1 M EDTA (ethylenediaminetetra-acetic acid) was added to dissolve the crystals, and the suspension was centrifuged and the supernatant analysed.

HYDROXYAPATITE CRYSTALS

Microcrystals of hydroxyapatite were prepared according to the method of Hayek and Newesely.²¹ Ca(NO₃)₂·4H₂O (7.9 g) was dissolved in water, alkalinised with ammonia to pH 12, and brought to a volume of 60 ml. (NH₄)₂HPO₄ (2.7 g) was dissolved in water, alkalinised to pH 12, and brought to a volume of 80 ml. Under vigorous stirring the latter solution was added to the Ca(NO₃)₂ solution. The precipitate was washed several times until ammonia free and then suspended in medium. The suspension was sonified briefly (20 s) and diluted to a stock suspension of 10 mg hydroxyapatite/ml. This method of preparation yields very small crystals of length 50–200 nm as determined by electron microscopy. The strong adsorption of proteins, such as LDH, to HyAp was overcome by dissolving the crystals after the experiment in 0.01 M EDTA. The final concentration of HyAp during the experiments was 100 µg/ml, unless otherwise indicated.

ENZYME RELEASE

Release of the cytoplasmic enzyme lactate dehydrogenase (LDH) was determined as a measure of plasma membrane damage (cytolysis). The release of granule associated enzymes, lysozyme and β-glucuronidase, was measured to establish a possible involvement of granule exocytosis in the enzyme release process. LDH was assayed by measuring the conversion of NADH into NAD⁺ (nicotinamide adenine dinucleotide) during the reaction of pyruvate to lactate. Lysozyme release was assayed by measuring the rate of lysis of *Micrococcus lysodeikticus* at pH 6.2, according to the method of Shugar.²² β-Glucuronidase was assayed by measuring the release of nitrophenol from *p*-nitrophenyl-β-D-glucuronide, as previously described.²³ Enzyme release is expressed as a percentage of maximal enzyme release after disruption of the cells with 0.2% Triton X-100.

NEURAMINIDASE TREATMENT

Neutrophils, 10^8 in 2 ml buffer pH 6.5, were

incubated with 10 units neuraminidase (Sigma Chemical Co, St Louis, MO, type V) for 30 min at 37°C. Then the cells were centrifuged; in the supernatant the liberated sialic acid was estimated according to Warren.²⁴ The cells were washed with medium and resuspended. Control cells were treated in the same way but without neuraminidase.

LIPOSOMES

Multilamellar liposomes were prepared from 100 µmol purified egg yolk lecithin, 75 µmol cholesterol, and 10 µmol of a charged lipid (dicethyl phosphate for negatively charged liposomes and stearylamine for positively charged liposomes). The lipids were dissolved in chloroform and spread as a thin film on the surface of a flask by rotary evaporation of the solvent in vacuo at 40°C. Subsequently, 5 ml of 0.3 M glucose solution was added, the mixture was left at 40°C for 30 min, and then the lipids were dispersed by vortexing for two minutes. The liposome suspension was dialysed extensively against 0.15 M NaCl. In the experiments 500 µl liposome suspension was mixed with HyAp or 0.15 M NaCl, brought to 1 mM Ca⁺⁺ and 1 mM Mg⁺⁺, and incubated in a final volume of 0.7 ml for one hour at 37°C. Then the reaction mixture was placed in a dialysis bag and dialysed against 1.5 ml 0.15 M NaCl for one hour. In the latter solution glucose was determined according to Bergmeyer *et al.*²⁵ The glucose measured in the control experiment (liposomes without crystals added) and which represents residual glucose, and glucose release in the absence of crystals, is subtracted from the glucose release. The release of glucose in excess of control is expressed as a percentage of glucose released from liposomes treated with 0.2% Triton X-100.

CYTOPLASTS

Cytoplasts were prepared from neutrophils according to Roos *et al.*²⁶ using discontinuous gradients of Ficoll containing cytochalasin B. Control neutrophils were treated with cytochalasin B, and the same washing procedure was followed to avoid differences as a consequence of residual cytochalasin B.

CHEMICALS

Polyvinylpyridine-*N*-oxide was obtained from K and K/ICN Pharmaceuticals (Plainview, NY). The following chemicals were from Sigma Chemical Co: poly-D-glutamic acid, mol. wt 66 000; poly-L-glutamic acid, mol. wt 95 000; heparin, 140 USP (United States pharmacopoeia) units/mg; dextran sulphate, mol. wt 500 000; rabbit albumin catalase and neuraminidase, type V from *Clostridium perfringens*. Pentex rabbit serum was from Miles

Fig. 1 Concentration dependence of HyAp crystal induced enzyme release from neutrophils. Values given are the mean value of three experiments. ●=LDH release; ○=lysozyme release; △=glucuronidase release.

Laboratories (Elkhart, IN). Cytochalasin A and cytochalasin B were from Aldrich Chemical Co (Milwaukee, WI). Egg yolk lecithin, cholesterol, dicethyl phosphate, and stearylamine were from Sigma Chemical Co; the lecithin was purified before use.

Results

Hydroxyapatite crystals caused a dose and time dependent release of enzymes from human neut-

rophils (Figs 1 and 2). This release referred both to cytoplasmic products such as lactate dehydrogenase (LDH), and to granule associated enzymes such as lysozyme and glucuronidase. The percentage of lysozyme release was considerably higher than that of LDH release, whereas the percentage of glucuronidase release was less. The release of LDH followed a sigmoid curve both in the time and the concentration curve.

Release of enzymes occurred in the absence of

Fig. 2 Time dependence of HyAp crystal induced enzyme release. Neutrophils were exposed to 100 µg HyAp/ml for a given time at 37°C. ●=LDH release; ○=lysozyme release; △=glucuronidase release.

Fig. 3 Effect of divalent cations on HyAp induced enzyme release. Neutrophils were exposed to 100 µg HyAp/ml in the presence of the indicated cations for 30 min at 37°C. The values given are the mean value of three experiments (SD). ▨=LDH release; □=lysozyme release.

divalent cations, but the presence of Ca^{++} had a strong potentiating effect, especially on lysozyme release (Fig. 3). A number of agents were tested that are known to interfere with the different cellular targets involved in phagocytosis and exocytosis. All these agents inhibited HyAp induced enzyme release (Table 1). The inhibition, however, was not complete, and for most agents was less than the degree of inhibition reported in published work for phagocytosis or exocytosis.

As previously shown with rabbit peritoneal granulocytes the strong hydrogen acceptor polyvinylpyridine-*N*-oxide has no influence on the

interaction between crystals and neutrophils. Several negatively charged polymers, such as polyglutamic acid, dextran sulphate, and heparin, strongly diminished HyAp induced enzyme release from neutrophils (Fig. 4). This applied both to LDH and lysozyme release. The same occurred with negatively charged proteins: albumin (and thus serum too) and catalase strongly inhibited HyAp induced enzyme release (Table 2); release of LDH was more strongly inhibited than release of lysozyme.

Because the preceding experiments suggested a role for positive charges in the cell-crystal interaction an attempt was made to localise these positive charges. Neutrophils or HyAp crystals were pretreated with the polyanion poly-D-glutamic acid, followed by centrifugation and washing to remove loosely bound polyanion. The pretreated cells or crystals were then exposed to non-treated crystals or cells respectively, and the degree of inhibition by the adherent polyanion was compared with the results of an experiment in which the polyanion was added before the incubation of cells with crystals (Table 3). The interaction between pretreated neutrophils and HyAp gave about the same enzyme release as that of control cells with HyAp. The interaction between cells and pretreated HyAp, however, gave almost no enzyme release and resembled the situation in which the polyanion was present during incubation.

Treatment of neutrophils with neuraminidase to remove the negatively charged sialic acid residues from the cell surface had little or no effect on the subsequent interaction between cell and HyAp

Table 1 Inhibition of HyAp-neutrophil interaction by several agents

Inhibitor	Enzyme release (%)	
	LDH	Lysozyme
—	62 (3)*	87 (3)
Cytochalasin A (5 µmol/l)	36 (4)	42 (4)
Cytochalasin B (5 µmol/l)	36 (3)	85 (3)
<i>p</i> -Bromophenacyl bromide (10 µmol/l)	20 (4)	26 (7)
<i>N</i> -Ethylmaleimide (100 µmol/l)	37 (3)	21 (4)
Iodoacetate (0.5 mmol/l)	45 (3)	47 (6)
Zn ⁺⁺ (1 mmol/l)	16 (4)	11 (3)
Co ⁺⁺ (1 mmol/l)	23 (4)	54 (2)
2-Deoxyglucose (20 mmol/l)	55 (2)	81 (6)

Cells were preincubated with the given concentration of inhibitor for 10 min at 37°C. Then 100 µg HyAp was added and the solution incubated for 30 min at 37°C.

*Values given are the mean of three experiments (SD).

Fig. 4 Effect of polyvinylpyridine-N-oxide and polyanions on HyAp induced enzyme release. Neutrophils were exposed to 100 μ g HyAp/ml, with or without the agents indicated, for 30 min at 37°C. Values given are the mean of three experiments (SD). P(D)GA=100 μ g poly-D-glutamic acid/ml; P(L)GA=100 μ g poly-L-glutamic acid/ml; dextr. sulph=100 μ g dextran sulphate/ml; heparin=100 μ g/ml; PVPNO=1000 μ g polyvinylpyridine-N-oxide/ml. ▨=LDH release; □=lysozyme release.

crystals: the enzyme release for neuraminidase treated cells was only slightly different from that of control cells (Fig. 5).

HyAp crystals were able to release glucose trapped in multilamellar liposomes (Fig. 6). This release occurred only when the liposomes were negatively charged; with positively charged liposomes the release of glucose in the presence of crystals was even less than control. The polyanion poly-D-glutamic acid completely annihilated the effect of HyAp on negatively charged liposomes (Table 4). With positively charged liposomes the polyanion gives a higher glucose release than with crystals alone, probably as a consequence of the interaction of negatively charged polyanion with the positively charged liposomes.

Table 2 Effect of some proteins on HyAp crystal induced enzyme release

Protein	Enzyme release (%)	
	LDH	Lysozyme
—	64 (2)*	83 (5)
Serum (50 μ g)	14 (3)	63 (4)
Serum (500 μ g)	6 (3)	23 (5)
Albumin (50 μ g)	15 (1)	49 (7)
Albumin (500 μ g)	3 (1)	14 (4)
Catalase (50 μ g)	66 (3)	77 (5)
Catalase (500 μ g)	21 (4)	68 (5)
Superoxide dismutase (50 μ g)	67 (2)	87 (6)
Superoxide dismutase (500 μ g)	66 (1)	78 (4)

Neutrophils were incubated with 100 μ g HyAp/ml, with or without the proteins as indicated for 30 min at 37°C.

*Values given are the mean of three experiments (SD).

Treatment of cytoplasts, prepared from neutrophils, with HyAp crystals showed that LDH release was only slightly less than that of control cells. Some lysozyme release could be measured.

Table 3 Effect of pretreatment of cells or crystals with poly-D-glutamic acid on HyAp induced enzyme release

	Enzyme release (%)		
	LDH	Lysozyme	Glucuronidase
Cells+HyAp	62 (4)*	85 (6)	44 (3)
Cells+PGA±HyAp	6 (1)	10 (2)	4 (2)
Cells (control)+HyAp	54 (3)	66 (3)	35 (2)
Cells (PGA)+HyAp	50 (2)	64 (4)	37 (2)
Cells+HyAp (control)	49 (5)	63 (6)	41 (3)
Cells+HyAp (PGA)	5 (1)	6 (2)	3 (1)

HyAp=100 μ g hydroxyapatite/ml; PGA=100 μ g poly-D-glutamic acid (mol wt 66 000) per 3×10^6 cells or per 100 μ g HyAp. Cells (PGA): 3×10^6 neutrophils were pretreated with 100 μ g PGA for 10 min at 37°C; then the cells were centrifuged, washed, and resuspended. Cells (control) underwent the same treatment but without PGA.

HyAp (PGA): 100 μ g HyAp was pretreated with 100 μ g PGA for 10 min at 37°C; then the crystals were centrifuged, washed, and resuspended. HyAp (control) underwent the same treatment but without PGA.

Cells and crystals, with or without pretreatment as indicated, were mixed and incubated for 30 min at 37°C; then EDTA was added, the suspension was centrifuged, and the supernatant analysed.

*Values given are the mean of three experiments (SD).

Fig. 5 Effect of pretreatment of neutrophils with neuraminidase on HyAp crystal induced enzyme release. Neutrophils were treated with or without neuraminidase as described in 'Materials and methods' and subsequently exposed to different concentrations of HyAp. ●=LDH release, control cells; ▲=LDH release, neuraminidase treated cells; ○=lysozyme release, control cells; △=lysozyme release, neuraminidase treated cells.

but the amount was very low, indicating that the cytoplasm has lost the major part of the granules, in accordance with data of other publications.²⁶ The membrane damaging effect of the crystals on cytoplasm could be antagonised by poly-D-glutamic acid (Table 5).

Discussion

The study of HyAp crystal induced enzyme release from cells is hampered by the fact that HyAp has a high and specific affinity for certain proteins,

including LDH, the release of which we used as a measure for plasma membrane damage. This difficulty was solved by dissolving the crystals with EDTA at the end of the experiment. Owing to the small size, the crystals were rapidly dissolved, and this procedure facilitated the subsequent experimental procedure.

HyAp crystals appear to have two different effects on neutrophils: (a) a membrane damaging effect, as can be deduced from the leakage of the cytoplasmic enzyme LDH; (b) an activating effect, resulting in

Fig. 6 Glucose release from negatively charged liposomes. Multilamellar liposomes prepared from lecithin, cholesterol, and dicethyl phosphate, and loaded with glucose, were exposed to various concentrations of crystals. Release of glucose was measured as described in 'Materials and methods'; the release of glucose in excess of control was expressed as a percentage of glucose release from Triton X-100 treated liposomes.

Table 4 *Hydroxyapatite crystal induced glucose release from liposomes*

	Glucose release (%)†
Negative liposomes, HyAp	51 (3)*
Negative liposomes, HyAp, PGA	0 (3)
Positive liposomes, HyAp	-9 (2)
Positive liposomes, HyAp, PGA	9 (1)

HyAp=2000 µg hydroxyapatite; PGA=1000 µg poly-D-glutamic acid.

*Values given are the mean of four experiments (SD).

†Glucose release in excess of control is expressed as a percentage of maximal glucose release obtained by treatment with Triton X-100.

exocytosis. Release of lysozyme is greater than that of LDH, which indicates that membrane damage is preceded by exocytosis of specific granules. The release of glucuronidase does not allow conclusions about a possible exocytosis of azurophilic granules. Because glucuronidase release is less than LDH release this can either indicate exocytosis of azurophilic granules, or cell disintegration which follows plasma membrane damage. The release of cellular constituents, either due to plasma membrane damage or exocytosis, results in the liberation of a large number of inflammation promoting constituents, and may thus lead to inflammation.

It has been shown previously that HyAp crystals are phagocytised by neutrophils.¹⁵ Phagocytosis is often accompanied by exocytosis, and this may explain the activating effect of the crystals. Cytochalasin B, which inhibits phagocytosis but not exocytosis, inhibits mainly LDH release but not lysozyme release from neutrophils. Inhibitors of phagocytosis and exocytosis, interfering with different targets such as metabolism (deoxyglucose, iodoacetate),

Table 5 *Hydroxyapatite crystal induced enzyme release from neutrophil cytoplasts*

	Enzyme release (%) from:		
	Control cells		Cytoplasts
	LDH	Lysozyme	LDH
—	7 (2)	14 (3)	10 (2)
100 µg HyAp	73 (4)	80 (5)	60 (4)
100 µg HyAp, 100 µg PGA	5 (4)	11 (4)	8 (2)

Control cells were treated with the same concentration of cytochalasin B as cytoplasts, and received the same washing procedure.

PGA=poly-D-glutamic acid, mol. wt 66 000.

*Values given are the mean of three experiments (SD).

sulphydryl groups (cytochalasin A, ethylmaleimide), phospholipase A₂ (bromophenacyl bromide), and unknown targets (Co⁺⁺, Zn⁺⁺), have modulating effects on HyAp induced enzyme release. It seems likely, therefore, that phagocytosis plays a part in the enzyme releasing effect of the crystals. This also explains the potentiating effect of Ca⁺⁺ because this ion is known to promote phagocytosis. The picture is incomplete, however, because enzyme release is usually not completely inhibited by these agents, which are known to block phagocytosis (and exocytosis) completely.²⁷⁻²⁹ This may be related to the small size of the crystals which enables them to be phagocytised without opsonisation.

In contrast with the action of urate crystals and silica,³⁰⁻³² hydrogen bonding interactions do not appear to play an important part in HyAp induced enzyme release, as is indicated by the lack of effect of polyvinylpyridine-N-oxide. Polyanions strongly inhibit HyAp induced enzyme release, which points to an important role for positive charges in the interaction. Cells and crystals have both positive and negative charges on their surface and the net overall charge is dependent on the pH, but even when the overall charge is negative the positive charges may play an important part in certain interactions. The experiment in which cells or crystals were pretreated with polyanion indicates that the polyanion has a high affinity for HyAp crystals and that the bound polyanion blocked the subsequent interaction with cells. It seems likely, therefore, that the positive charges which are playing a predominant part in the cell-crystal interaction are located on the crystal surface.

This mechanism may have important consequences for the effect of HyAp in vivo. As shown by our in vitro experiments, some negatively charged proteins have a strong inhibiting effect on the cell-crystal interaction. A comparable modulating effect by negatively charged proteins may be exerted in vivo in places where HyAp crystals are deposited, and induce inflammation.

Sialic acid residues make an important contribution to the negative charge on the cell surface. Removal of these sialic acid residues with neuraminidase has little effect on the cell-crystal interaction. It seems therefore unlikely that an interaction between the positive charges on the crystals and the negatively charged sialic acid residues play an important part in the cell-HyAp interaction.

The experiments with liposomes show that HyAp crystals are able to disrupt lipid bilayer membrane when these are negatively charged. Polyanions annihilate the disrupting effect. These results indicate that here too positive charges on the crystals play a predominant part and that the lipid section of

the membrane may be the target of the HyAp crystals.

For the disruption of phagocytes by urate crystals a mechanism has been proposed in which the crystals exert their effect after phagocytosis by an attack on the phagolysosome membrane.^{31,32} The results with cytoplasts show that the membranes of these neutrophil derived structures can be damaged by HyAp without the involvement of granules; in this regard they resemble human erythrocytes, which are readily haemolysed by HyAp crystals.³³ Phagocytosis nevertheless appears to have a role in HyAp induced enzyme release as can be deduced from the effect of cytochalasin B, Ca^{++} , and inhibitors, but the nature of this role is not easy to establish. It may be that phagocytosis results in the formation of a protein free region in the membrane, thus allowing an easier attack by other crystals on these lipid regions. Such a mechanism does not exclude an attack of the crystals taken up by phagocytosis on the phagolysosome membrane. The relative contribution of these processes to the final release of enzymes remains to be established.

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