

Hydroxyapatite and urate crystal induced cytokine release by macrophages

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SUMMARY Destructive osteoarthritis is characterised by rapidly progressive joint destruction associated with intra-articular deposition of hydroxyapatite crystals. The possible role of such crystals in the pathogenesis of this condition was investigated by testing the ability of hydroxyapatite crystals to stimulate the production of bone resorbing activity from mouse peritoneal macrophages. Urate crystals were used for comparison. Culture supernatants were tested for bone resorbing activity using the mouse calvarial bone resorption assay, for interleukin 1 using a standard lymphocyte activation assay, and for prostaglandin E₂ by radioimmunoassay. Culture supernatants from macrophages incubated with hydroxyapatite crystals contained dialysable bone resorbing activity, high concentrations of prostaglandin E₂, but no interleukin 1 like activity. The production of the bone resorbing agent was prevented by culturing macrophages with hydroxyapatite crystals in the presence of indomethacin. By contrast, culture supernatants from macrophages incubated with urate crystals contained bone resorbing activity, which was only partly removed by dialysis, and interleukin 1 like activity. The latter was shown to be increased in culture supernatants from macrophages incubated with urate crystals in the presence of indomethacin, while production of bone resorbing activity was partially inhibited. It is considered that the bone resorbing activity liberated from macrophages stimulated by hydroxyapatite crystals can be explained by the presence of prostaglandin E₂ alone, whereas the activity liberated by urate crystals is due to both prostaglandin E₂ and interleukin 1.

Key words: destructive osteoarthritis, bone resorption, interleukin 1.

Particles containing apatite are frequently found in osteoarthritic joints,¹⁻³ especially in those with radiological evidence of severe and rapid joint destruction.⁴⁻⁶ It has been suggested that such crystals are involved in the pathogenesis of osteoarthritis.⁷⁻⁸ Hydroxyapatite crystals can induce acute inflammatory reactions in human skin⁹ and synovitis after injection into dogs' knee joints.¹⁰ No association has been found, however, between either the clinical assessment of synovitis or the number of synovial fluid cells and the presence of hydroxyapatite crystals in human synovial fluid samples.¹¹⁻¹² The apparent lack of an association between inflammation and hydroxyapatite deposition is emphasised in destructive osteoarthritis, where there is no influx

of inflammatory cells despite large synovial effusions containing numerous crystalline particles.²⁻³

Alternatively, there is evidence that crystals may contribute to joint damage by mechanisms dependent on resident cells in the synovium. For example hydroxyapatite crystals have been shown to stimulate the release of destructive enzymes, such as collagenase and neutral proteases, from synovial cells in culture¹³ and can stimulate cultured synovium to release prostaglandin E₂.¹⁴ In addition, urate crystals are known to stimulate macrophages to produce interleukin 1,¹⁵⁻¹⁶ which is one of a number of cytokines that can stimulate bone and cartilage resorption.¹⁷⁻¹⁸ Recently, we described bone resorbing activity in the synovial fluids of patients with destructive osteoarthritis in association with hydroxyapatite crystal deposition¹⁹ and suggested that hydroxyapatite crystals stimulate the local production of a bone resorbing agent from synovial

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macrophages. This led us to examine the ability of hydroxyapatite crystals to stimulate the release of bone resorbing activity from macrophages and to examine whether this activity is due to interleukin 1. This paper describes the finding and partial characterisation of a bone resorbing agent in culture supernatants from mouse peritoneal macrophages stimulated with hydroxyapatite.

Materials and methods

PREPARATION OF MACROPHAGES

C57B1/6 black mice (10–16 weeks old) were killed by ether and washed with 70% ethanol. Eagle's minimum essential medium (5 ml) with Earle's salt (Gibco) containing 5 U/ml heparin (Evans) was injected intraperitoneally into each mouse and their abdomens massaged gently. The peritoneal fluid was collected, the cells centrifuged and resuspended in medium. The cell suspension was layered on top of a 56% percoll gradient and the gradients centrifuged (2500 g for 10 minutes). The macrophage rich bands were removed, the cells centrifuged and resuspended in medium with Earle's salts supplemented with 2 mM L-glutamine (Gibco), 10% 309 fetal calf serum (low endotoxin, batch number K223901A Gibco, Europe), 200 U/ml penicillin (Glaxo), and 100 µg/ml streptomycin (Evans). The cell concentration was adjusted to 2×10^6 cells/ml and cultured in 24 well plastic plates for three to four hours to allow the macrophages to adhere, then washed three times with fresh medium to remove any contaminating lymphocytes.

ADDITION OF CRYSTALS TO MACROPHAGES

Hydroxyapatite and urate crystals were synthesised according to standard methods.^{20 21} Their chemical purity was checked by infrared spectroscopy. They were also examined by light microscopy, and samples containing particles of similar morphology to those found in human joint disease were used. Urate crystals were needle shaped crystals of monosodium urate monohydrate, between 2 and 10 µm long; the hydroxyapatite was partially carbonated, rounded clumps of crystals, with a diameter of between 0.1 and 5 µm. Aliquots (10 mg) of crystals were sterilised by autoclaving for one hour at 180°C, suspended in 10 ml sterile phosphate buffered saline, and sonicated for three seconds. They were added to the adherent macrophages at concentrations between 50 and 1800 µg/ml and cultured for 24 hours. Culture supernatants were harvested, centrifuged, filter sterilised, and assayed for bone resorbing activity, interleukin 1, and prostaglandin E₂ concentrations both before and after dialysis. Dialysis was carried out against medium for 48 hours with 6000–8000 MW cut off dialysis membranes (Spectrapor dialysis membranes, Spectrum Medical Industries Inc, Los Angeles).

In some experiments 10^{-6} M indomethacin and 10^{-6} M hydrocortisone were added to the culture, and the culture supernatants were assayed both before and after dialysis.

BONE RESORPTION ASSAY

Bone resorbing activity was assessed quantitatively using the mouse calvarial system.²²

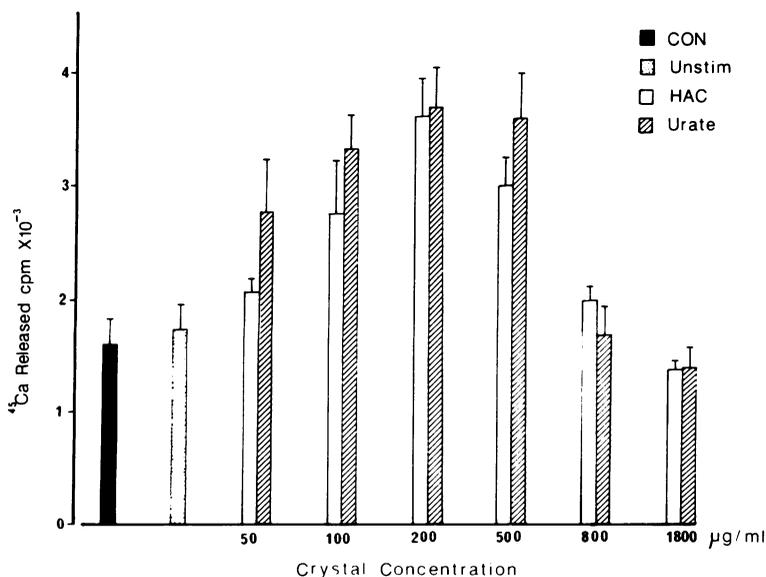


Fig. 1 Bone resorbing activity released from macrophages stimulated with increasing concentrations of hydroxyapatite or urate crystals for 24 hours. The results are the mean cpm (SD) of the amount of ⁴⁵Ca released from treated bones compared with that released from controls (CON) for four experiments. Each experiment was done in quadruplicate. Macrophage-crystal supernatants were tested at a final concentration of 10%. Unstim = ⁴⁵Ca release from bones treated with supernatants of unstimulated macrophages; HAC = hydroxyapatite crystals.

INTERLEUKIN 1 ASSAY

The lymphocyte activation assay of Gery *et al* was performed as described elsewhere.²³

RADIOIMMUNOASSAY FOR PROSTAGLANDIN E₂
 Prostaglandin was assayed by radioimmunoassay using a commercial kit (Amersham International) which determines 13,14-dihydro-15-ketoprostaglandin E₂ in the range 43–690 pg/tube.

STATISTICAL ANALYSIS

Student's *t* test was used to compare the control and experimental groups in both assays.

Results

BONE RESORBING ACTIVITY

Hydroxyapatite and urate crystals were added to macrophages at increasing concentrations. After 24 hours the culture supernatants were tested for bone resorbing activity at a final concentration of 10%. Figure 1 shows the results obtained. Macrophages stimulated with hydroxyapatite or urate crystals produced increasing amounts of bone resorbing activity. The effect was maximal at a crystal concentration of 200 µg/ml in the macrophage-crystal cultures, but it decreased at concentrations above this level.

Supernatants from macrophages stimulated with either 200 µg/ml of hydroxyapatite or urate crystals

were tested by the bone resorption assay at final concentrations of 1%, 10%, and 15%. Figure 2 shows the results from macrophages stimulated with hydroxyapatite crystals; more ⁴⁵Ca was released from bones treated with 10% culture supernatant than with 1% culture supernatants, though there was no further increase with 15% culture supernatants. A similar pattern of results was obtained with supernatants from macrophages stimulated with urate crystals except that the highest ⁴⁵Ca release was obtained with 15% culture supernatants.

Culture supernatants from macrophages treated with hydroxyapatite or urate crystals were assayed for bone resorbing activity before and after dialysis. The results show that for hydroxyapatite supernatants dialysis removed all the bone resorbing activity (Fig. 3). In contrast, although dialysis of supernatants from macrophages cultured with urate significantly reduced the level of activity compared with that before dialysis (*p*<0.01), some activity remained.

In an attempt to characterise the factor or factors released from macrophages after stimulation with crystals the effect of indomethacin on the ability of macrophages to produce bone resorbing agents was examined. Table 1 shows that addition of indomethacin to macrophages inhibited the release of bone resorbing activity in response to both hydroxyapatite and urate crystals. To determine whether the inhibitory effect of indomethacin was due to a direct

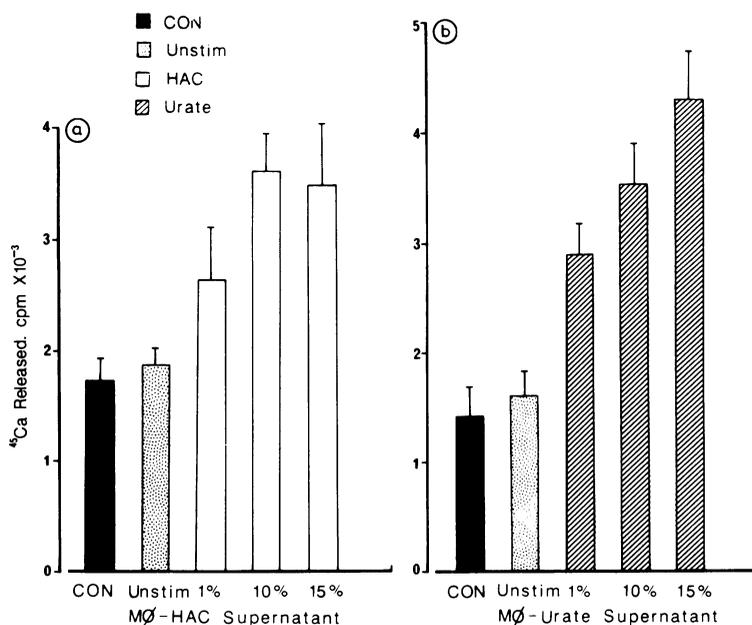


Fig. 2 (a) Bone resorbing activity in supernatants from macrophages (MØ) stimulated with 200 µg/ml hydroxyapatite crystals (HAC). The results are the mean cpm (SD) of the amount of ⁴⁵Ca released from treated bones compared with that released from controls (CON) and from bones treated with supernatants from unstimulated macrophages (Unstim) for four experiments. Each experiment was done in quadruplicate. (b) Bone resorbing activity in supernatants from macrophages (MØ) stimulated with 200 µg/ml urate crystals. The results are the mean cpm (SD) of the amount of ⁴⁵Ca released from treated bones compared with that released from controls for four experiments. Each experiment was done in quadruplicate.

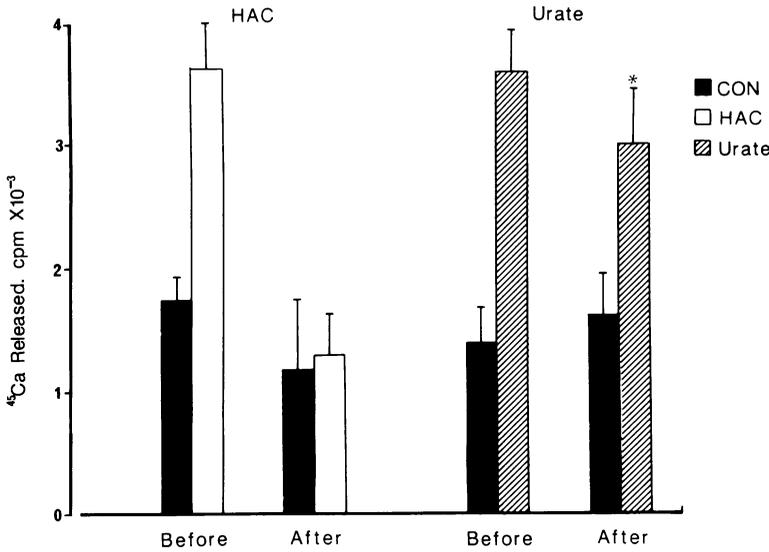


Fig. 3 Effect of 10% supernatants from macrophages stimulated with 200 µg/ml hydroxyapatite (HAC) or urate crystals on the release of ⁴⁵Ca from cultured bones before and after dialysis. The results are expressed as the mean cpm (SD) of the amount of ⁴⁵Ca released from treated bones compared with that from controls (CON) for four experiments. Each experiment was done in quadruplicate. * p<0.01.

Table 1 Bone resorbing activity in supernatants from macrophages cultured with 200 µg/ml hydroxyapatite or urate crystals with or without addition of indomethacin to the culture. Values are mean (SD)*

Addition	Before dialysis	After dialysis
HAC† alone	2.07 (0.21)	1.09 (0.04)
HAC+indomethacin	0.99 (0.07)	1.12 (0.16)
Urate alone	2.55 (0.24)	1.85 (0.04)
Urate+indomethacin	1.05 (0.08)	1.67 (0.25)

*The results are those obtained before and after dialysis of the macrophage-crystal supernatants and are expressed as the ratio of ⁴⁵Ca released from treated bones to that released from controls. Each experiment was done in quadruplicate. Supernatants were tested at a final concentration of 10%.
†HAC=hydroxyapatite crystals.

effect on the calvarial assay or whether indomethacin prevented the production of bone resorbing activity by macrophages, culture supernatants were dialysed to remove the indomethacin and retested in the bone resorption assay. Table 1 shows that most of the bone resorbing activity in the supernatants of macrophages cultured with urate crystals was restored after dialysis. Dialysis failed to restore the activity of supernatants from macrophages stimulated with hydroxyapatite crystals, however. Similar experiments were performed using hydrocortisone. In these, inhibition of activity was also observed both with urate and hydroxyapatite crystals, but this was not reversed by dialysis.

INTERLEUKIN 1 ACTIVITY

Hydroxyapatite and urate crystals were added at

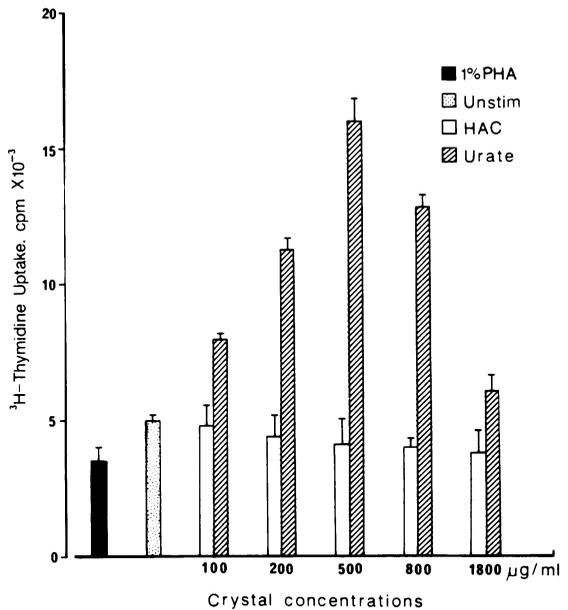


Fig. 4 Interleukin 1 like activity in supernatants from macrophages stimulated with increasing concentrations of either hydroxyapatite (HAC) or urate crystals. The interleukin 1 activity is expressed as the uptake of thymidine and is the mean cpm (SD) of six experiments. Each experiment was done in quadruplicate. Supernatants were tested at a final concentration of 3%. PHA=thymocytes stimulated with phytohaemagglutinin alone; Unstim=thymocytes stimulated with supernatants of unstimulated macrophages; CON=controls.

increasing concentrations to macrophages. After 24 hours the culture supernatants were tested for interleukin 1 like activity at a concentration of 3%. The results show that hydroxyapatite crystals failed to stimulate the release of interleukin 1 from macrophages (Fig. 4). By contrast, urate crystals stimulated the release of interleukin 1 like activity from macrophages; this latter effect increased with increasing crystal concentrations, reaching a maximum at 500 $\mu\text{g/ml}$.

The effect of indomethacin (and hydrocortisone)

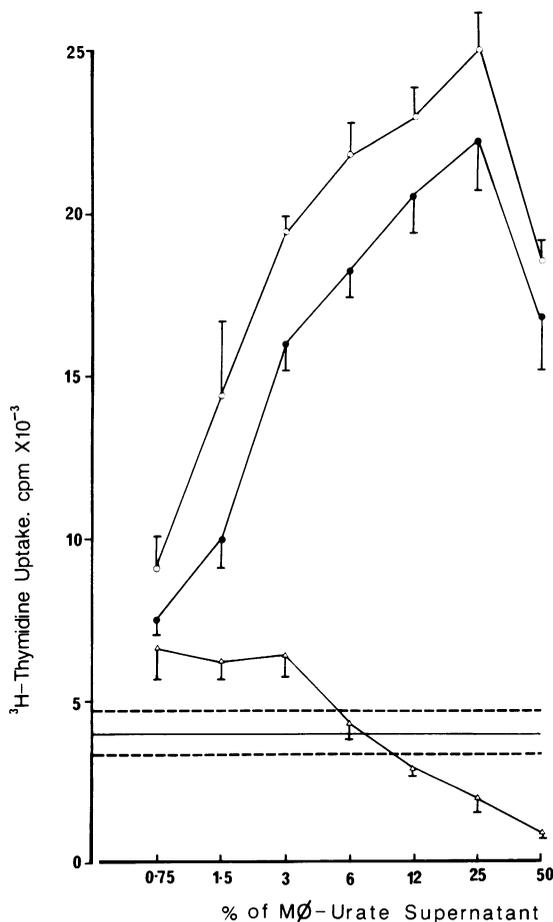


Fig. 5 Interleukin 1 activity in supernatants from macrophages ($M\phi$) stimulated with 500 $\mu\text{g/ml}$ urate alone (●), urate plus indomethacin (○), and urate plus hydrocortisone (△). The results are the mean cpm (SD) of thymidine uptake for four experiments. Each experiment was done in quadruplicate. The supernatants were tested at increasing concentrations. The dashed lines represent 1 SD on either side of the mean given by phytohaemagglutinin alone.

on the release of interleukin 1 activity from macrophages cultured with urate crystals (500 $\mu\text{g/ml}$) was investigated. Figure 5 shows that supernatants from macrophages stimulated with urate in the presence of indomethacin had significantly ($p < 0.01$) higher concentrations of interleukin 1 than supernatants from macrophages stimulated with urate crystals alone. Culture supernatants from macrophages incubated with urate crystals in the presence of hydrocortisone contained no interleukin 1 activity, and the activity was not restored by dialysis to remove the hydrocortisone.

Macrophages cultured with hydroxyapatite or urate crystals for 24 hours were examined by electron microscopy. Both types of crystal were found to have been phagocytosed by macrophages, though crystals were also found in association with the surface membrane.

MEASUREMENT OF PROSTAGLANDINS

The prostaglandin concentrations in supernatants from macrophages stimulated with 200 $\mu\text{g/ml}$ hydroxyapatite or urate crystals for 24 hours were tested by radioimmunoassay. High concentrations (above 800 pg/ml of prostaglandin E_2) were found in both supernatants. After dialysis prostaglandin was no longer detected in the supernatants.

Discussion

These results show that macrophages incubated with hydroxyapatite crystals release bone resorbing activity but not interleukin 1 like activity. For the following reasons we attribute the bone resorbing activity to prostaglandin E_2 . Culture supernatants active in the bone resorption assay had high concentrations of prostaglandin E_2 , which is the most potent bone resorbing agent among the prostaglandins.²⁴ Dialysed supernatants were inactive and contained no detectable prostaglandin E_2 . In addition, supernatants from macrophages incubated with hydroxyapatite crystals in the presence of the prostaglandin synthetase inhibitor indomethacin lacked bone resorbing activity. The fact that indomethacin blocked production of bone resorbing activity rather than inhibiting the bone resorption assay was inferred from the observation that the activity in the culture supernatants was not restored by removal of the drug by dialysis.

In contrast with macrophages stimulated with hydroxyapatite crystals, macrophages incubated with urate crystals released both bone resorbing and interleukin 1 activity. In view of the known ability of interleukin 1 to stimulate bone resorption^{17,18} it seems likely that some of the bone resorbing activity in the culture supernatants must be due to the

presence of interleukin 1. This is further supported by the findings that both significant bone resorbing activity and interleukin 1 activity were retained after dialysis of culture supernatants, and that hydrocortisone, which inhibits interleukin 1 production by macrophages,²⁵ prevented the production of urate induced bone resorbing activity. Because some activity was lost during dialysis, however, we surmise that production and release of a second mediator of bone resorption, probably prostaglandin E₂, is also induced by urate crystals. High concentrations of prostaglandin E₂ were found in the culture supernatants of urate stimulated macrophages, and the production of bone resorbing activity was reduced but not removed by indomethacin. It should be noted that indomethacin actually enhanced urate induced interleukin 1 release, presumably by inhibition of the synthesis of prostaglandin E₂, which is a potent inhibitor of interleukin 1 production.²⁶

Our results are consistent with previous reports that hydroxyapatite crystals are potent stimulators of prostaglandin E₂ from rheumatoid and normal canine synovial cells and rabbit articular chondrocytes.^{14, 27} In vitro it has been shown that hydroxyapatite crystals are more potent than pyrophosphate crystals in stimulating release of prostaglandin E₂ from human synovial cells, even at the lower concentrations tested.

Our data are also consistent with those from other groups, who found that urate but not hydroxyapatite crystals release interleukin 1 like activity from phagocytic cells in culture.¹⁶

These and other data therefore indicate that urate crystals stimulate the release of several mediators from phagocytic cells, including prostaglandin E₂ and interleukin 1, whereas hydroxyapatite crystals release prostaglandin E₂ but no interleukin 1. This raises two questions: firstly, what mechanisms dictate the differential effects of different particles on mediator release from cells, and, secondly, whether these 'in vitro' findings are relevant to the associated human joint disease.

Particles, including hydroxyapatite crystals, are rapidly removed from joints, probably via synovial lining phagocytic cells.²⁸⁻³⁰ Removal, however, may depend on particle size and protein coating, factors which are known to affect cell-particle interactions.³¹ Once phagocytosed, crystals may dissolve intracellularly, and there is evidence that calcium containing particles then induce different responses, perhaps via the intracellular calcium flux resulting from dissolution.³² In our experiments only pure urate and hydroxyapatite were used. They were autoclaved to remove endotoxin or other contaminants that can affect interleukin 1 production. The hydroxyapatite and urate crystals were of

comparable size and morphology to those seen in human synovial fluid samples, and to those used in other experiments on the inflammatory potential of these particles.^{21, 22, 31, 33} Previous experiments have shown that individual crystals or clumps of crystals in this size range are readily phagocytosed.^{34, 35} The surface area presented to the cell by the hydroxyapatite and urate crystals is different, however, raising the possibility that the differences in cytokine output are quantitative, rather than representing a different quality of response. One argument against this is the fact that the quantitative response of other systems is similar when comparable doses and crystal sizes are used.^{9, 31} Furthermore, if the data have relevance to human disease, use of particles with the right morphology is probably more appropriate than attempting to match them for surface area. The interaction of cells and particles in vivo may still be quite different because of the possible adsorption of different proteins and inflammatory mediators to the surface of the particles, rather than the total area presented to the cell.³¹

Chronic tophaceous gout and 'apatite associated destructive arthritis' are both connected with bone loss and apparent bone resorption.³⁶ It is obviously tempting to conclude that this is mediated by crystal induced release of factors such as interleukin 1 and prostaglandin E₂. Quite different factors may be involved, however, and in osteoarthritis the crystals may be more important as markers of different types of disease process rather than as initiators of pathological changes.³⁴ The danger of extrapolating these in vitro findings to the in vivo situation is exemplified by our previous data showing that interleukin 1 like activity is present in some synovial fluids from patients with destructive osteoarthritis and extensive hydroxyapatite crystal deposition.¹⁹ In the light of the findings presented in this paper it seems likely that this interleukin 1 activity is not stimulated by the presence of hydroxyapatite crystals. Nevertheless, the demonstration that crystals can induce factors which stimulate bone resorption, and the identification of such factors in patients' synovial fluid, is direct evidence for a sequence by which particles have the potential to damage bone.

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