Hydroxyapatite phagocytosis by human polymorphonuclear leucocytes

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SUMMARY Hydroxyapatite crystals were incubated with human polymorphonuclear leucocytes and samples examined by light and electron microscopy after 3, 8, 30, and 120 minutes' incubation. Phagocytosis of crystals occurred at 3 minutes and increased with incubation. Degranulation of neutrophils, loss of cytoplasmic density, and cell necrosis were greatest in cells mixed with apatite and increased with time. Avid *in vitro* phagocytosis of hydroxyapatite crystals lends further support to the potential of these crystals as causes of human arthritis.

Apatite crystals have been implicated in periarthritis (McCarty and Gatter, 1966) and have recently been suggested as a cause of crystal induced arthropathy (Dieppe *et al.*, 1976; Schumacher *et al.*, 1977; Amor *et al.*, 1977). Previous studies have shown that monosodium urate crystals and calcium pyrophosphate dihydrate crystals incubated with human white blood cells are phagocytosed and lead to different degrees of necrosis of the phagocytic cells (Schumacher and Phelps, 1971; Schumacher *et al.*, 1975). In this report we describe our results from incubation of hydroxyapatite crystals with human white blood cells and the sequential changes observed after crystal phagocytosis.

Materials and methods

Hydroxyapatite crystals (type I) suspended in $0 \cdot 001$M phosphate buffer at pH $6 \cdot 8$ were obtained from Sigma Chemical Co., St. Louis, Missouri, Crystals were present in $0 \cdot 4$–15μm clumps; the hydroxyapatite was heated for 3 hours at 180°C to eliminate any possible pyrogenic material.

Human polymorphonuclear leucocytes (PMNL) were separated from heparinised venous blood by dextran sedimentation (Klein, 1958) and suspended in the resulting plasma-heparin-dextran at a final concentration of 13 400 cells per mm³. As in previous experiments with urates and calcium pyrophosphates the suspension in addition to PMNLs contained

small numbers of monocytes, eosinophils, platelets, and erythrocytes.

1 mg of hydroxyapatite crystals was added to 4 of eight 160×13 mm tubes containing $1 \cdot 0$ ml aliquots of the cell suspension. All tubes were centrifuged at 350 *g* for $2 \cdot 5$ minutes and then incubated at 37°C. Paired tubes with and without crystals were incubated for 3, 8, 30, and 120 minutes. After each timed incubation aliquots were removed for light microscopic examination. 8 ml of $\frac{1}{2}$ strength Karnovsky's fixative was then added to each tube for electron microscopy. After brief fixation specimens were diced into 1×1 mm pieces, fixed in $\frac{1}{2}$ strength fixative diluted 1:1 with $0 \cdot 1$ cacodylate buffer at pH $7 \cdot 4$ for 4 hours, postfixed in cold Palade's osmium veronal for 2 hours, dehydrated with alcohol, and embedded in Epon 812. Ultrathin sections were prepared on an LKB ultramicrotome. Grids were examined on a Zeiss EM 10 electron microscope. The percentage of cells with phagocytosis of crystals and with cells necrosis was estimated by counting 50–100 cells from each tube.

Four wet smears were made from each of the aliquots taken before fixation and examined by phase and compensated polarised light microscopy. The percentage of cells containing hydroxyapatite was calculated after examining 400 cells from each tube.

Results

Three minutes after the crystals were added to the cell syspension phagocytosis could be appreciated by both light and electron microscopy (Table 1).

Table 1 *Hydroxyapatite incubation with human polymorphonuclear cells*

% cells with crystals	Time of incubation in minutes			
	3	8	30	120
	%	%	%	%
Light microscopy	3	6	8	10
Electron microscopy	10	12	49	51
% cell necrosis				
Control	12	9	10	19
Cells mixed with crystals	12	10	38	36

As noted in the Table, estimated percentages of cells containing phagocytosed crystals tend to increase with time. By light microscopy apatite clumps, whether intra- or extracellular, appeared globular or rod like with irregular margins. Most crystal clumps were not birefringent, but occasional clumps did have weakly positive or negative birefringence.

By electron microscopy, after 3 minutes, polymorphonuclear leucocyte processes were seen enfolding extracellular apatite. Other PMNLs examined at 3 minutes already had some apatite entirely within cell vacuoles. Both rod-like clumps and small clusters of crystals continued to be seen in the process or being enfolded and phagocytosed at all time periods examined (Figs. 1–3).

There was some evidence of degranulation of
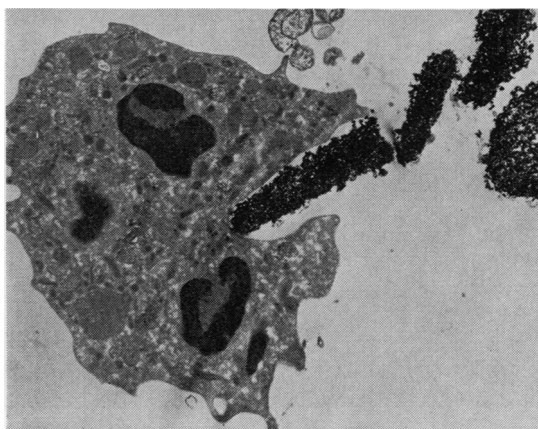
Fig. 1 *Dark rod-like clump of hydroxyapatite crystals being enfolded by polymorphonuclear cell processes. Electron micrograph,* × *7100, 30 minutes' incubation.*

neutrophils and loss of cytoplasmic density in all tubes at 3 minutes and throughout the study: this was greatest in the cells mixed with apatite and appeared to increase with time. Cells mixed with apatite without identified phagocytosis of crystals
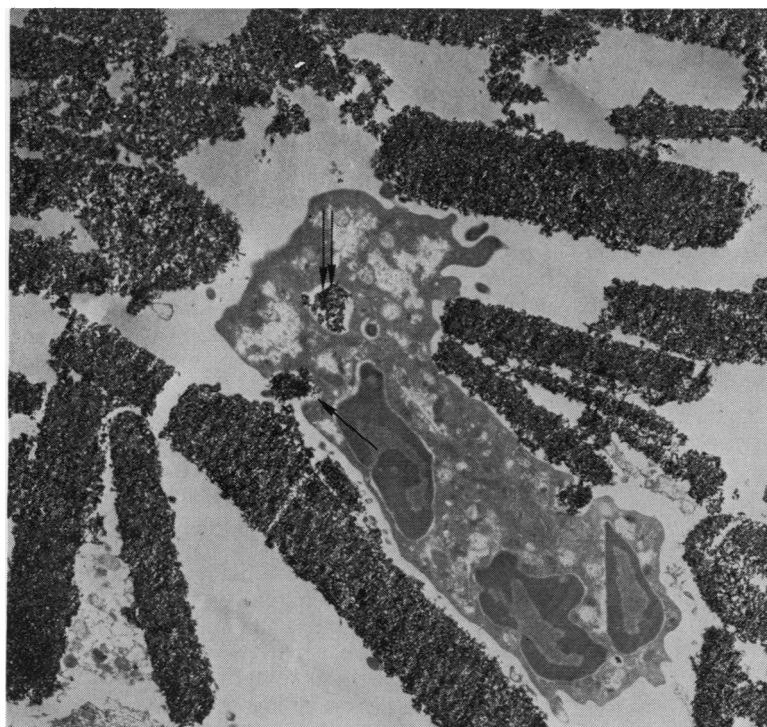
Fig. 2 *Large rod shaped extracellular clumps of hydroxyapatite with smaller clumps in a well defined phagosome (double arrow) and in process or being enfolded by cell processes (single arrow).*
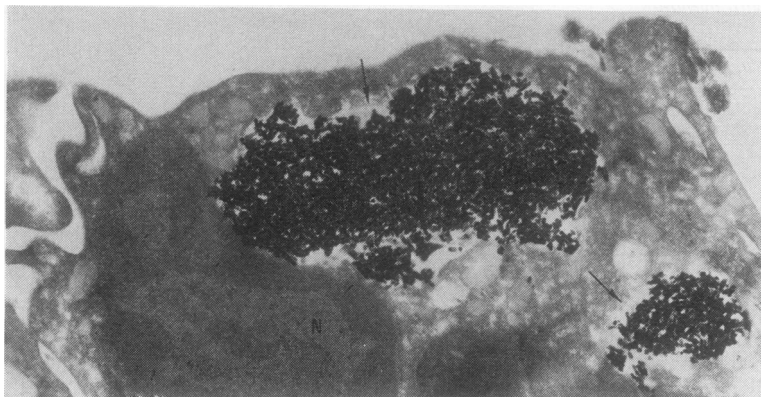
Fig. 3   *Clump of apatite crystals in phagosome of polymorphonuclear cell. Clear vacuoles adjacent to the phagosome probably are a result of degranulation. Phagosomal membranes are indistinct at several areas (arrows) N=nucleus. Electron micrograph × 20 000, 8 minutes' incubation.*

also had prominent degranulation but one could not be certain that an adjacent section of the cell would not be shown to contain crystals.

Dense bodies were only infrequently seen in phagosomes with crystal clumps. Phagosomal membranes were sometimes not well defined around apatite clumps, but similar indistinct phagosomal membranes were also occasionally seen in controls, presumably at least in part due to tangental cutting.

Only disruption of the cell with loss of external cell membranes was considered evidence of cell necrosis for this study. Some cell necrosis was identifiable by electron microscopy at 3 minutes, probably reflecting cell damage during separation. The percentage of necrotic cells estimated by electron microscopy increased with time, reaching 35% at 120 minutes in specimens mixed with crystals compared to only 19% in controls (Table 1). Where crystal clumps were seen within necrotic cells, they were generally large clumps (Fig. 4). No external lysis of cell membrane was seen related to direct contact from an extra cellular crystal clump.

Monocytes, eosinophils, and platelets were also present, and these also occasionally phagocytosed crystals. Monocytes and eosinophils containing crystals did not show necrosis or loss of cytoplamic density.

Discussion

These *in vitro* studies show that clumps of hydroxyapatite crystals are readily phagocytosed by human polymorphonuclear leucocytes and also by other cells. The smallest clumps of crystals would not be detected by light microscopy and probably account for the lower percentage of cells identified as containing crystals by light microscopy than by electron microscopy. Small clumps, especially when not birefringent, would also be difficult to identify in

synovial or bursal fluids. Thus, the possible importance of apatite crystals in joint diseases has only been widely recognised after electron microscopy studies. It should be noted that sodium urate (Schumacher *et al.*, 1975b) and calcium pyrophosphate crystals (Honig *et al.*, 1977) in human gout and pseudogout are occasionally so small that they also are recognised only by electron microscopy.

Although cells mixed with hydroxyapatite did show greater necrosis than control cells, and necrosis did seem to be associated with crystal phagocytosis, we can not yet be certain about mechanisms involved. Urate crystals appear to lyse phagosomal membranes (Schumacher and Phelps, 1971; Wallingford and McCarty, 1971; Weissman, 1971; Schumacher, 1977). Some loss of phagosomal membranes was seen in these experiments with apatite, but further studies will be needed to establish the role of this type of cell injury with apatite. Clumps of crystals of several microns in length seemed to be associated with most necrotic cells, so that there may be an optimal clump size for phagocytosis and cell damage. The possible role of crystal size on sodium urate and calcium pyrophosphate phagocytosis and cell damage has been examined (Schumacher *et al.*, 1975a).

Apatite crystals from human joint effusions have often been surrounded by electron dense material (Schumacher, 1977; Schumacher *et al.*, 1977). That was not seen in this *in vitro* system even though there was some serum in the incubating medium. Dense material coating urate crystals from gouty joints has been shown to contain immunoglobulins (Hasselbacher and Schumacher, 1976). Immunoglobulin or other materials bound to the surface of urate crystals in vivo may increase the avidity of phagocytosis (Kozin and McCarty, 1976).

Microcrystals resembling hydroxyadatite and shown by electron-probe analysis to contain calcium
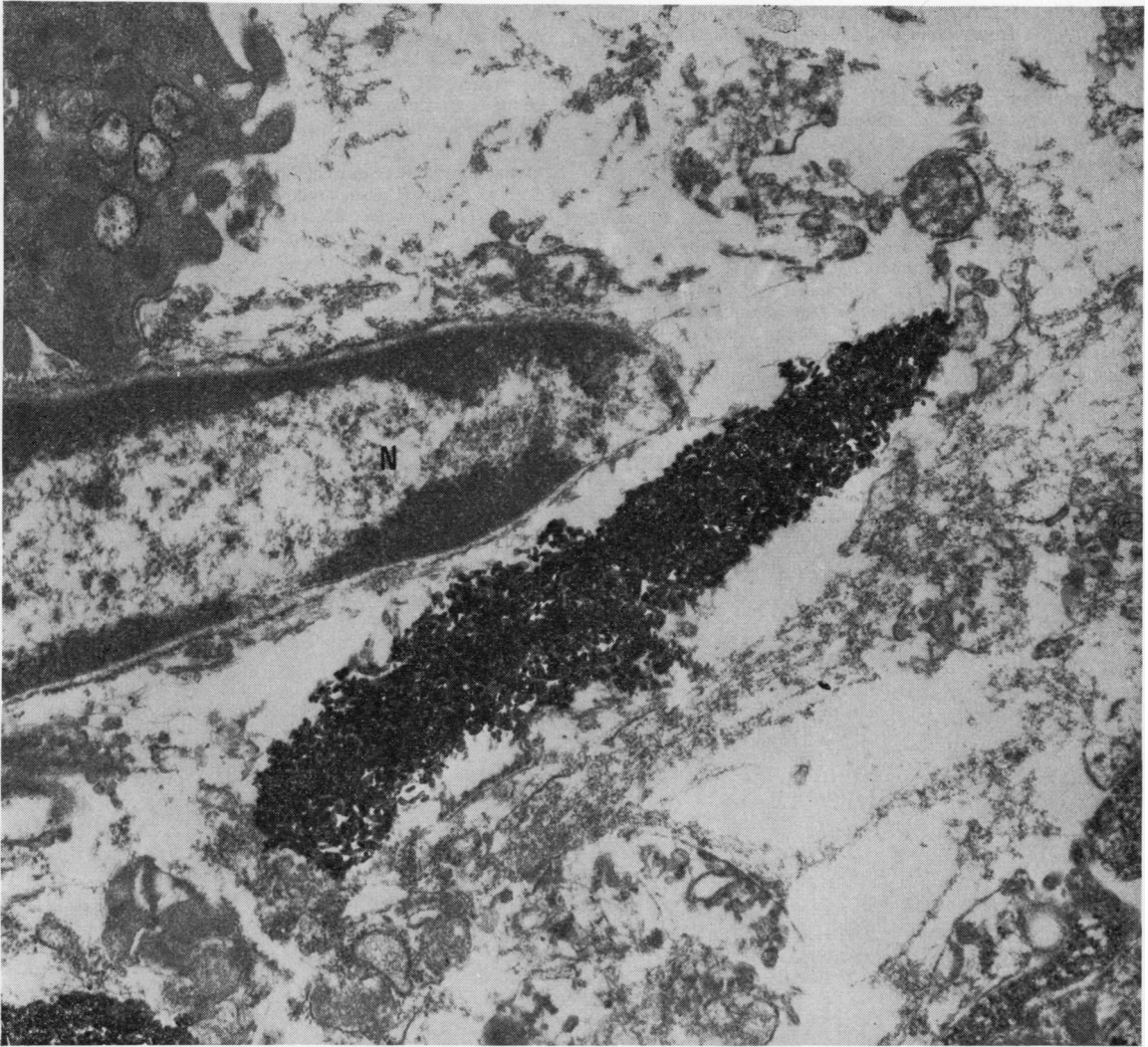
Fig. 4   *Necrotic polymorphonuclear cell with large clump of apatite crystals. Most external cell membrane and phagosomal membrane is lysed. N=nucleus. There is an intact cell in the upper left-hand corner.*

and phosphorous in a ratio consistent with apatite have been demonstrated in synovial fluid leucocytes (Schumacher *et al.*, 1977). Hydroxyapatite crystals are associated with an acute synovitis when injected into canine knee joints. (Schumacher *et al.*, 1976) and cause acute inflammation when injected into human skin and rat pleura (Dieppe *et al.*, 1976). Our study demonstrating ready *in vitro* phagocytosis of hydroxyapatite crystal clumps adds further support to the comparability of these clumps with urate and calcium pyrophosphate crystals as potential causes of human arthritis.

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