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The nature of iron deposits in haemophilic synovitis

An immunohistochemical, ultrastructural and X-ray microanalytical study

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Summary. Using a computerized electron-probe X-ray microanalytical technique to measure phosphorus/iron ratios we have defined the iron saturation of ferritin in vitro from prepared ferritin standards of known iron loading. This technique has been applied to the study of 5 haemophilic synovial membranes. At light microscope level the distribution and relationship of iron/ferritin were defined using Perls' reaction and an immunoperoxidase technique respectively. The synovia from all cases contained intra and extra-cellular deposits of Perls' positive material which were granular in nature in the most superficial synovial cells. There were increasing numbers of pheomorphic (1–12 μm diameter ovate bodies in the deeper synovial layers. Immunoperoxidase ferritin staining produced a strongly positive reaction in the granular material but the ovate bodies were negative with the exception of some peripheral staining. X-ray microanalysis showed the granular material to be highly iron saturated ferritin and the ovate bodies to be almost pure iron. We suggest that iron saturated ferritin in the synovial membrane could increase/perpetuate inflammation by promoting lipid peroxidation.

Key words: Haemophilia – Synovitis – Iron – Ferritin – X-ray-microanalysis

Introduction

In macrophages and certain epithelial cells ingestion of erythrocytes is followed by their digestion in phagolysosomes and liberation of iron from haemoglobin (Fedorko et al. 1973; Zeligs 1977). In phagocytic cells this process is normally associated with ferritin (Fineberg and Greenburg 1955; Richter 1961; Chu and Fineberg 1969; Hay and Jacobs 1981), at first in the cytoplasm, but later as aggregations in secondary lysosomes or multivesicular bodies. A similar process may be expected to take place in the synovial cells in haemophilic synovitis where repeated haemorrhages into the joint

cavity are a prolific source of erythrocytes and indeed, ultrastructurally, the synovium is found to contain typical iron filled lysosomes (Ghadially et al. 1976; Stein and Duthie 1981). Ultrastructural and electron-probe microanalytical studies of the synovial membrane from cases of haemophilic synovitis (Ghadially et al. 1976; Stein and Duthie 1981) and ultrastructural studies of experimental haemarthrosis in the rabbit (Roy and Ghadially 1966; Ghadially and Roy 1969; Ghadially et al. 1974) have failed to demonstrate the presence of ferritin in association with the synovial iron deposits. Recent studies have reported elevated levels of synovial fluid ferritin in inflammatory joint disease (Blake and Bacon 1981) and shown the ability of ferritin to promote lipid peroxidation in vitro (Gutteridge et al. 1983) with the potential for disruption of lysosomal membranes and release of hydrolytic enzymes in vivo. The chronicity of haemophilic synovitis associated with extensive synovial iron deposits has led us to re-investigate a potentially pathogenic iron/ferritin relationship in this disease using immunohistochemistry, ultrastructural morphology and electron-probe X-ray microanalysis.

Materials and methods

1. Electron-probe microanalysis and ultrastructural studies

Synovial tissue was obtained either at synovectomy or replacement arthroplasty from five cases of haemophilic synovitis (Table 1). Because of the scarcity of material, many of the tissue samples had been previously fixed in 10% formal-saline. This fixative was found to give acceptable ultrastructure if the synovium was cut into 1 mm^3, extensively washed in 0.1 M phosphate buffer (pH = 7.2) and post fixed in 2% aqueous osmium tetroxide. One synovectomy sample obtained during the course of this study was fixed specifically for electron-microscopy in 2.5% glutaraldehyde in cacodylate buffer (0.1 M, pH = 7.4) followed by 2% aqueous osmium tetroxide. Glutaraldehyde and formaldehyde specimens were also processed without oxmication for X-ray microanalysis. All specimens were embedded in Spurr's resin (Spurr 1969) following dehydration in ethanol. For morphological studies, ultrathin sections were mounted on copper grids, stained with lead citrate/uranyl acetate and viewed in a Siemen's Elmiskop 102 electron microscope at an accelerating voltage of 80 Kv. Thicker sections (0.5 μm) mounted on copper grids were carbon coated and analysed for phosphorus (P) and iron (Fe) using a Jeol JEM – 1200 EX electron microscope with computerized X-ray microanalytical facilities. Ferritin samples of known iron loading were also analysed under the same conditions as the tissue, samples being dried directly on carbon coated formvar films on copper grids. The relationship between P and Fe was expressed as a ratio following measurement of relevant

Table 1. Details of patients with haemophilic arthritis. All patients had clinical and radiological evidence of arthritis. X-ray changes included joint narrowing, erosions and cyst formation

Case	Age (years)	Factor deficiency	Factor (%)	No. of arthritic joints	Sites of specimens
1	32	IX	0	6	Knee
2	41	VIII	1	6	Elbow
3	25	VIII	2	6	Knee
4	31	VIII	4	5	Knee
5	23	VIII	0	6	Knee

peak heights from the computer print-out (Myagkaya and De Bruijn 1982). Structurally, ferritin is a complex macromolecule with protein (apoferritin) subunits forming a shell around a core containing a variable amount of iron oxyhydroxide (Fe OOH) x and some phosphorus. The P:Fe ratios are, therefore, directly related to the structure of the molecule, particularly the quantity of iron in the core.

2. Histochemistry and Immunohistochemistry

Sections of 10% formal saline fixed, wax embedded sections were prepared using standard histological techniques. Deposits of trivalent iron in the synovial membranes were demonstrated using Perls' prussian blue technique (Perls 1867). Ferritin was localised using the peroxidase anti-peroxidase technique (Sternberger et al. 1970), sections being briefly digested with trypsin initially, to give improved staining (Curran and Gregory 1977). Endogenous peroxidase was blocked with 0.5% hydrogen peroxide in methanol and sections were pre-treated with normal swine serum. The three antisera used were monospecific rabbit anti-human ferritin, swine anti-rabbit IgG and rabbit anti-peroxidase/peroxidase complex. Peroxidase activity was demonstrated by the method of Graham and Karnovsky (1966).

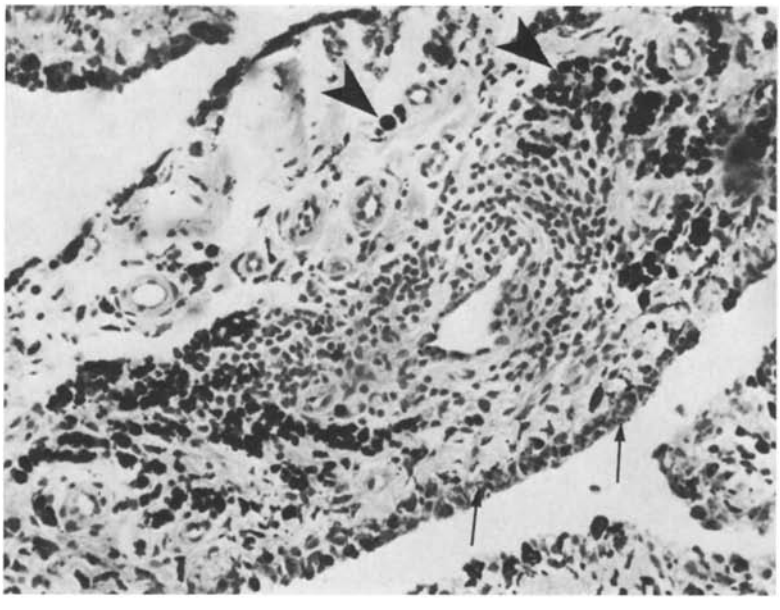
Results

1. Histochemistry and Immunohistochemistry of synovial iron deposits

The synovia from all five cases of haemophilic arthritis contained extensive intra- and extra-cellular deposits of Perls' positive (ferric) iron. These deposits were present in the form of granular siderosomes in the synovial lining cells, but in the sub-intimal macrophages, tended to occur as ovate bodies varying in diameter from 2–12 μm (Fig. 1). Most such bodies were intra cellular but some appeared to be free within the sub-intimal connective tissue. Immunoperoxidase staining for ferritin was strongly positive in the granular siderosomes of the synovial lining cells, and there was also "peppering" of positive material in the cytoplasm of many cells (Fig. 2). This discreet ferritin is also seen with the Perls' reaction as an indistinct but definite cytoplasmic blueness. The ovate bodies in the deeper synovial tissue did not stain for ferritin, though some had strong peripheral reactivity.

2. Ultrastructural Studies

In all cases, the majority of synovial lining cells of both types A and B contain highly electron-dense siderosomes, pleomorphic lysosomal bodies reacting positively with Perls' stain. Much of the electron-dense material is granular in nature, but amorphous areas of extreme density are frequently present within the granular deposits (Fig. 3). In type A cells, the siderosomes appear to be randomly distributed throughout the cytoplasm, whereas in type B cells they are located peripherally. There is, however, a preponderance of type A synovial cells in all the synovia studied. Some of the deposits are apparently derived from mitochondria and contain recognizable cristae



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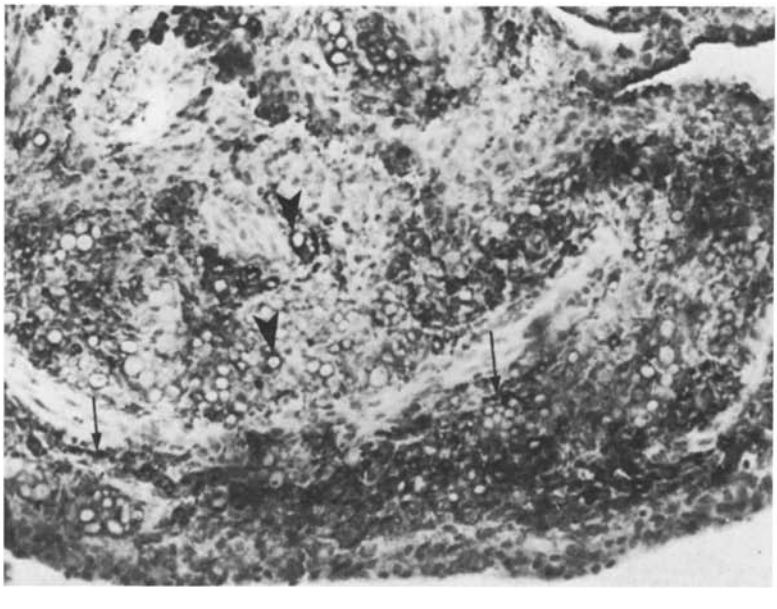

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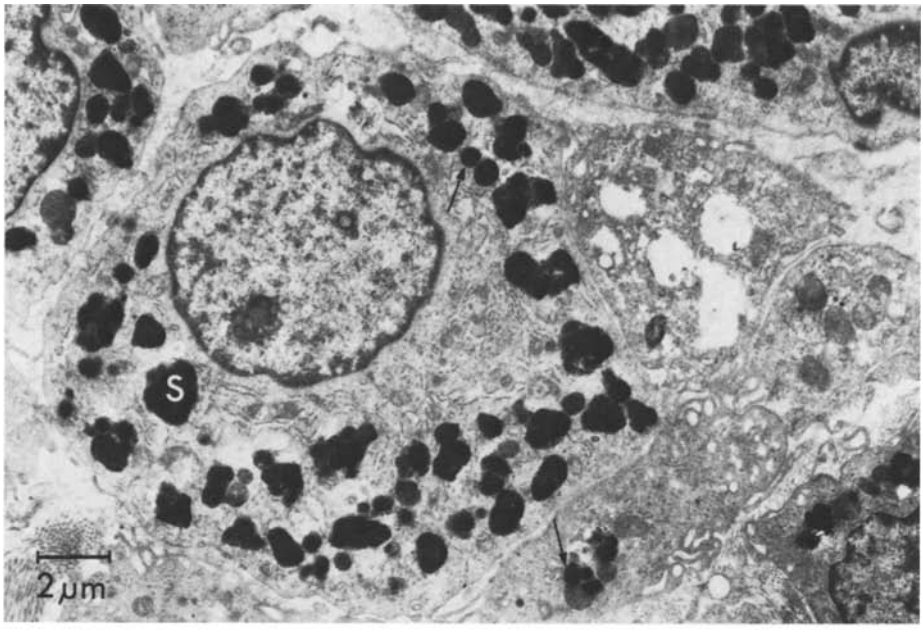
Fig. 1. *Case 2.* Perls' positive granular siderosomes are present in the synovial lining cells (*arrows*). There are prominent Perls' positive ovate bodies in the fibrous tissue beneath the synovial lining cell later (*arrow heads*). Magnification ×160

Fig. 2. *Case 2.* The peroxidase-antiperoxidase technique demonstrates ferritin as a brown reaction product (*arrows*) in association with siderosomes and scattered throughout the cytoplasm of many cells. The Perls' positive ovate bodies are negative but some show peripheral reactivity (*arrow heads*). Magnification ×400



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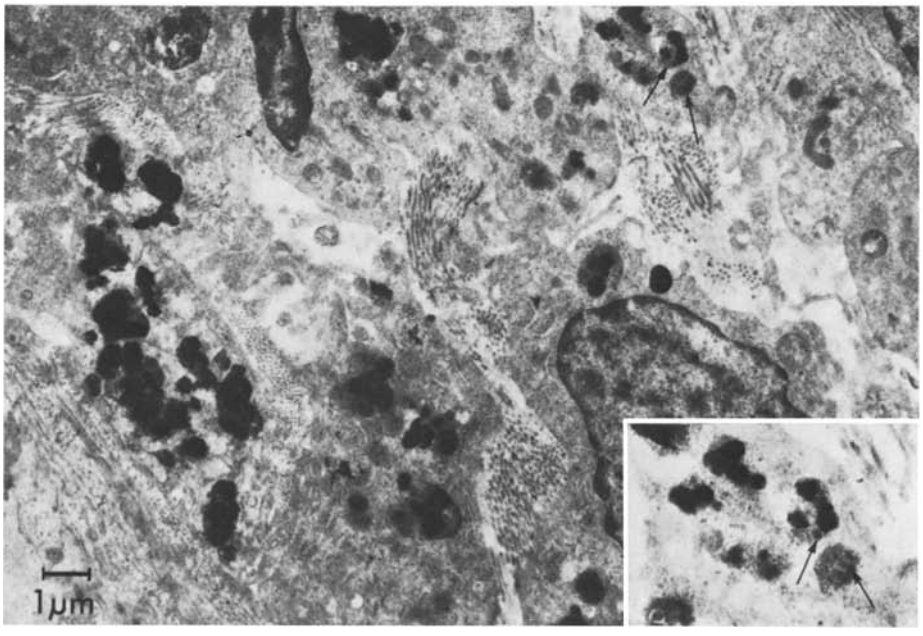

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Fig. 3. *Case 4.* Electron micrograph of synovial lining cells most of which are of type A and contain electron-dense pleomorphic lysosomal bodies (siderosomes) reacting positively with Perls' stain (*S*). Much of the electrondense material is granular but occasional amorphous areas of extreme density are present (*arrows*). The large cell is of type B with peripherally distributed siderosomes

Fig. 4. *Case 4.* Electron micrograph of synovial lining cells showing numerous siderosomes some of which appear to be of mitochondrial origin and contain recognizable cristae (*arrows*). These are shown in higher magnification in inset (*bottom right*)

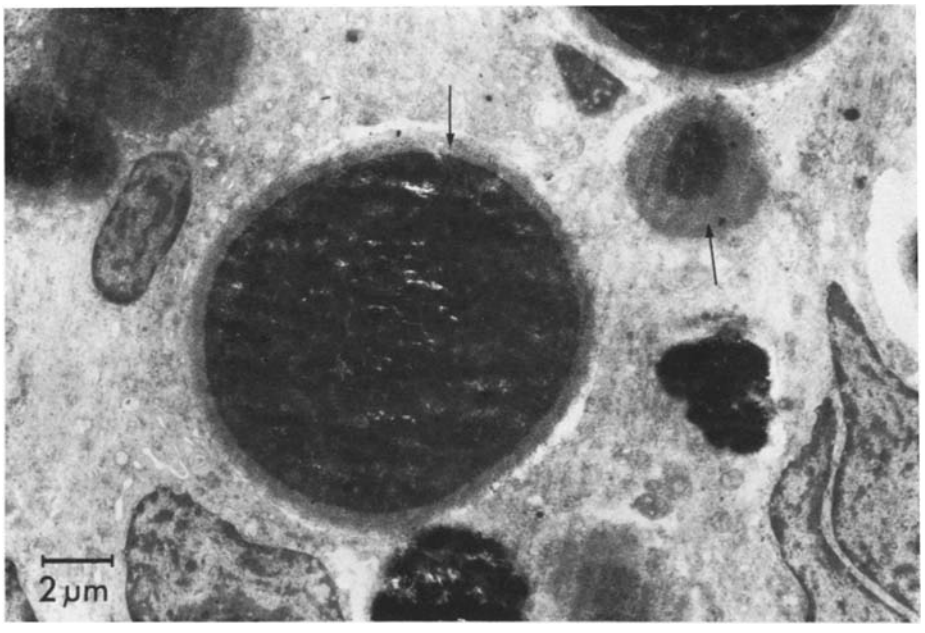

Fig. 5. *Case 3.* Electron micrograph of sub-intimal connective tissue showing typical ovate bodies with an electron dense core and a concentrically layered structure. The granular material around the periphery is probably ferritin (*arrows*) as demonstrated immunohistochemically in Fig. 2. Cells were frequently in a degenerating state

Table 2. Results of electron-probe X-ray micro-anaalysis of ferritin samples showing phosphorus (P)/iron (Fe) ratios at different iron loading of the ferritin molecule

P/Fe ratio	Iron loading of ferritin (mols Fe/mole protein)
1:3.3	4,230
1:2.5	4,150
1:1.6	4,000
1:1.2	3,950
1:0.9	2,430
1:0.8	1,920
1:0.6	900
1:0.2	710
1:0.1	500

(Fig. 4). The Perls' positive ovate bodies in the sub-intimal cells are layered structures with material of least electron-density peripherally (Fig. 5). They are frequently associated with granular bodies of low electron density. Many of the cells containing large or multiple ovate bodies were disrupted with loss of intra-cellular organelles. There was a marked increase in size of siderosomes in the deeper synovial cells when compared with those in the lining cell layer.

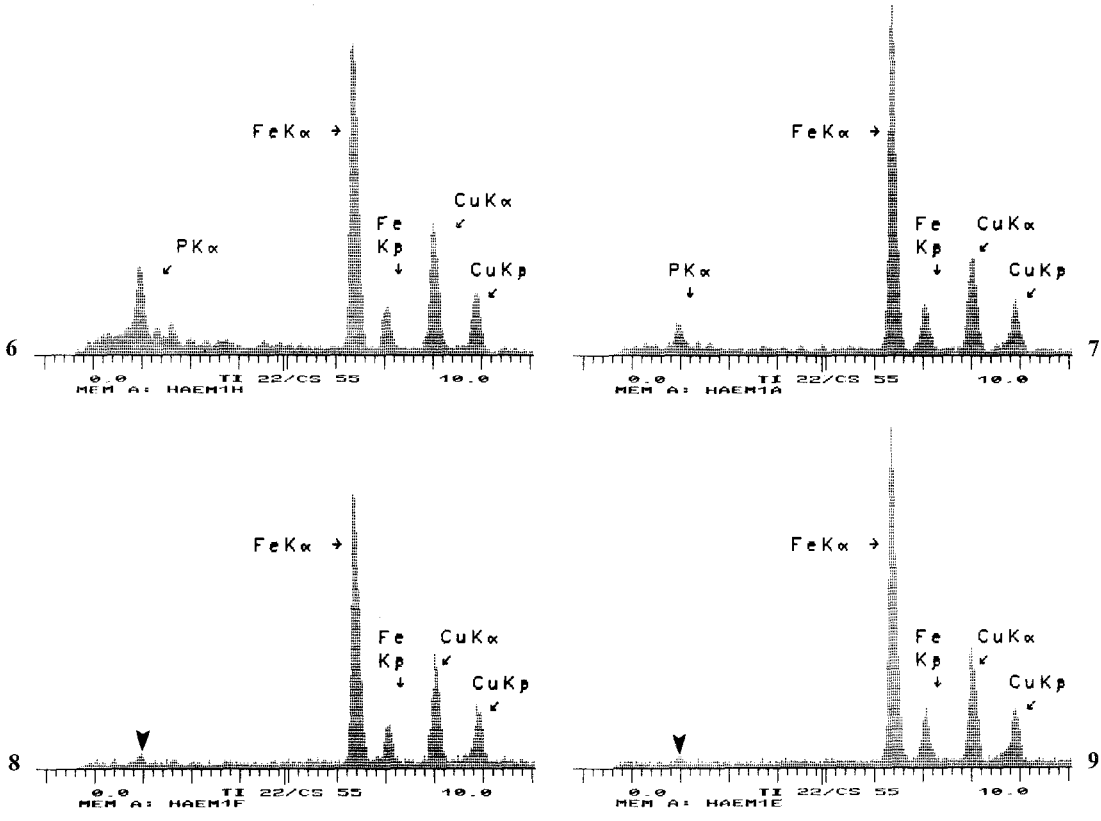

Fig. 6. *Case 3.* Electron probe X-ray microanalysis computer printout of a typical siderosome analysis in the synovial lining cells. Defined peaks for phosphorus (*PKα*) and iron (*Fe Kα*) are clearly seen. The copper (*Cu Kα*) peak is derived from the copper grid which supports the sections during analysis. The phosphorus to iron (*P/Fe*) ratio is 1:4, indicating the presence of highly iron saturated ferritin (see Table 2)

Fig. 7. *Case 3.* Typical X-ray microanalysis of a deep synovial siderosome. Similar peaks to those in Fig. 6 are present, but the *PKα* is much less prominent —P/Fe ratio 1:12. The siderosomes at this level contain some non-ferritin iron as the P/Fe ratio is much greater than our most iron saturated ferritin samples

Fig. 8. *Case 4.* X-ray micro-analysis of the less electron-dense outer shell of an ovate body. The PKα peak (*arrow head*) is sufficiently above background to be significant, and correlates with the presence of a small amount of ferritin in this region

Fig. 9. *Case 4.* X-ray microanalysis of the inner core of an ovate body. There is a well defined *Fe Kα* peak, but the *PKα* (*arrow head*) is not significantly above background. This region is, therefore, composed entirely of hydrated ferric oxide

3. X-ray microanalytical studies

Electron-probe X-ray microanalysis of ferritin samples of known iron content showed that the phosphorus:iron (P:Fe) ratio calculated by measuring relevant peak heights was directly related to the iron loading of the ferritin molecule (Table 2). The same procedure applied to tissue sections demon-

strated that the granular siderosomes had X-ray emission spectra typical of highly iron saturated ferritin with a mean P:Fe ratio of 1:4 (Fig. 6). Siderosomes from the deeper synovium (Fig. 7) and particularly the ovate bodies had a different type of emission spectrum with a great deal more iron present in relation to phosphorus, Mean P:Fe ratios varying from 1:10 to 1:33 for siderosomes and peripheral regions of the ovate bodies (Fig. 8). The cores of the bodies, however, consisted of iron with virtually no detectable phosphorus (Mean P:Fe ration 1:86) (Fig. 9).

Discussion

The outstanding pathological feature of the haemophilic synovium is the massive deposition of iron in the synovial cells. It is likely that these deposits are derived from the breakdown of erythrocytes phagocytosed from repeated bleeding into the joint space, many of the ultrastructural features of the haemophilic synovitis being reproducible in animal joints by injection of erythrocytes (Roy and Ghadially 1966; Ghadially and Roy 1969; Ghadially et al. 1974). The majority of the iron deposits would be expected in the synovial A cells which have a phagocytic role (Ball et al. 1964; Barland et al. 1964; Cochrane et al. 1965; Norton and Ziff 1966; Shannon and Graham 1970; Chamberlain et al. 1972), a fact confirmed by Stein and Duthie (1981) and ourselves. We also note that the synovium was hypercellular and that the majority of cells were of type A – presumably a response to the presence of haem products in the synovium. The phagocytosed red cells are subsequently incorporated into lysosomal bodies which are of two main types (Roy and Ghadially 1969; Simon and Burke 1970), siderosomes containing haemosiderin derived from degraded haemoglobin and myelinoid bodies composed of osmiophilic membranes derived by hydration of erythrocyte membrane lipid residues. Ghadially et al. (1976) describe both simple and compound siderosomes in their study of the haemophilic synovial membrane, but the majority of siderosomes in our cases were made up of particulate matter of varying size and electron density and were of the compound type. There was also decreased synovial cellularity in the case described by Ghadially, most of the siderosome containing cells being modified type B synoviocytes, contrary to the classical concept of the non-phagocytic B cell (Barland et al. 1964). Many of the B cells in our cases also contained siderosomes, but these were always distributed around the cell periphery rather than randomly as in type A synoviocytes, presumably reflecting a difference in the phagocytic process. Studies of horseradish-peroxidase injected into guinea-pig synovium indicate that this material is taken into synovial B cells by a process of micropinocytosis and remains for long periods in the peripheral cell cytoplasm (Chamberlain et al. 1972). A cells were found to incorporate the marker by a larger scale type of phagocytosis.

The sub-intimal synovial tissue in all our cases contained relatively few cells but the siderosomes were very much larger due to movement and coalescence of iron rich material down through the synovial cell layers. This could take place by movement of cells downwards from the synovial

lining cell layer (Roy and Ghadially 1966) coupled with transfer of material from one cell to another possibly by re-phagocytosis following cell degeneration and disintegration. Degenerating cells were commonly found in the sub-intimal region in our cases and were also described by Schumacher (1973). Some of the largest siderosomes had a lamellated structure indicating several distinct cycles of phagocytosis. Synovial lining cells frequently contained degenerating cell organelles, particularly mitochdrondria, in proximity to siderosomes which frequently contained identifiable cristae (Fig. 4). Richter has suggested that siderosomes may be derived from mitochrondria (Richter 1967), but as secondary lysosomes, the siderosomes could have acquired the cristae by intake of cell debris. The organelle disruption may also be a reflection of free radical damage in the presence of ferritin iron (Willson 1978; Crichton 1979).

In patients with rheumatoid arthritis, iron deposition in the synovial membrane is well documented (Muirden and Senator 1968; Goldie 1970; Kumar and Garg 1974) and such deposits, usually in the form of haemosiderin, may arise from phagocytosed red cells leaking from granulation tissue (Collins 1951; Muirden and Senator 1968). Haemosiderin deposition appears to be associated with an increase in ferritin production (Richter 1957) which is most marked in synovial A cells (Muirden 1966). Synovial cells in vitro also have the ability to absorb haemoglobin iron from the culture medium and convert it to ferritin (Sturgeon and Shoden 1969). Such an association has not been described previously in haemophilic synovitis, although in all our cases, ferritin was demonstrable in large quantities both free in the synovial cell cytoplasm and within siderosomes. Using electron-probe X-ray microanalysis we have found that the ferritin in the lining cell siderosomes is highly iron saturated (P:Fe, 1:4, Fig. 6) when compared with the emission spectra of a series of ferritin samples of known iron saturation (Table 2). In vitro highly iron saturated ferritin promotes lipid peroxidation (Gutteridge et al. 1983) with the potential to disrupt lysosomal membranes and release hydrolytic enzymes with associated tissue damage. This process may also take place in vivo in the ferritin laden haemophilic synovium producing a chronic inflammation. There is certainly a high level of hydrolytic enzyme activity, notable cathepsin D (Hilgartner 1973) in the synovial tissues and synovial fluid.

The amount of ferritin iron decreases in cells deeper in the synovium, the large siderosomes containing iron with little associated phosphorus (P:Fe, 1:10–1:33, Fig. 8) and the large ovate bodies containing virtually no detectable phosphorus (P:Fe, 1:82, Fig. 9) and no immunocytochemical reactivity for ferritin (Fig. 2). The protein moiety of the ferritin is, therefore, gradually removed by proteolysis, the core material being added to the haemosiderin iron of the siderosome. This iron may be in the form of ferric hydroxide ($Fe(OH)_2$) as suggested by Sturgeon and Shoden (1969) or more likely the stable hydrated ferric oxide ($Fe_2O_3 \times H_2O$) (Ghadially et al. 1976).

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