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Immunohistochemical findings in rheumatoid nodules

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Summary. Eighteen nodules from patients with rheumatoid disease were studied histologically and immunohistochemically. A continuum of microscopic changes was observed with varying degrees of fibrinoid necrosis, mononuclear cell infiltration and healing by fibrous scarring. In two cases there was focal evidence of arteritis. Fibrin was plentiful in the necrotic areas of nodules. Small amounts of immunoglobulin were identified in plasma cells and as irregular extracellular deposits in and around areas of necrosis. In a single small vein abnormal IgG was detected.

Mononuclear cells surrounding areas of necrosis stained strongly with antisera to ferritin and a cytoplasmic macrophage antigen, stained variably with muramidase (lysozyme) and negatively with alpha-1 antitrypsin antibodies. Perls' stain for ferric iron was almost entirely negative and ultrastructural x-ray microanalysis indicated that the cytoplasm of these cells were entirely free of iron. These findings confirm the chronic inflammatory nature of rheumatoid nodules but provide no support for the view that they originate in areas of vasculitis. A relative lack of cytoplasmic antiprotease along with a strong expression of ferritin appears to be a characteristic feature of macrophages in rheumatoid tissue.

Key words: Rheumatoid nodule – vasculitis – immunohistochemistry – ferritin – fibrin – antiproteases.

Introduction

On clinical, histological and immunological grounds rheumatoid nodules remain one of the most enigmatic lesions in rheumatology. The microscopic appearances are distinctive yet, apart from a passing resemblance to granu-

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loma annulare of the skin and uncommon prostatic lesions (Mies et al. 1984) the lesion has no histological counterpart.

In this report we describe the immunohistochemical findings in paraffin embedded sections of 18 nodules and ultrastructural observations on a further two. The objectives were to assess the importance of immunoglobulin and fibrin deposition in necrotic areas and around blood vessels. In view of our interest in iron metabolism in rheumatoid disease particular attention was directed to the antigenic characteristics of the mononuclear infiltrates. The iron and apoferritin content of these cells were assessed using both immunohistochemical and ultrastructural techniques.

Materials and methods

1. Patients studied. Twenty nodules were removed from 19 patients, 8 males and 11 females, median age 57, range 24–80 years. Each satisfied ARA criteria for classical or definite rheumatoid disease and had had symptoms for 4–27 years (mean 8 years). All had positive tests for rheumatoid factor in the serum and had received, or were receiving, second line drug treatment such as gold or penicillamine. Ten of the nodules were biopsied from the forearm or elbow and 5 from the hand and 5 from other sites. The mean maximum dimension of the nodules, as measured in the surgical pathology laboratory was 18 mm (range 10–40 mm).

2. Immunohistochemical methods. All biopsies were fixed in 10% neutral buffered formol saline, processed into paraffin and cut at 5 μm thickness. The immunoperoxidase technique was then used to study the distribution of the heavy chains of IgA, IgG and IgM, the C3 component of complement, fibrin, albumin, alpha-1 anti-trypsin, the lysosomal enzyme muramidase, the iron transport protein transferrin and the intracellular storage protein apoferritin. In representative cases antisera to kappa and lambda light chains of immunoglobulin, Clq and C4 components of complement and an antibody (S22) directed against a 200,000 Daltons molecular weight glycoprotein present in the cytoplasm of macrophages (Isaacson and Jones 1983) were also used.

In most cases an indirect immunoperoxidase technique was satisfactory. For each individual case the most appropriate dilutions of the primary rabbit anti-human antibodies was determined by titration. These varied, for example, from 1/50 for ferritin, 1/200 to 1/500 for IgG and 1/200 to 1/800 for IgM. The peroxidase labelled secondary swine anti-rabbit antibody was used at 1/50. For optimal staining of fibrin, muramidase and the macrophage membrane antigen (S22) a modification of the unlabelled antibody-peroxidase-anti peroxidase (PAP) method of Sternberger was used (Mepham et al. 1979). Sections were pre-treated with a freshly prepared solution of 0.05% trypsin at 37° C for between 20 and 30 min. The primary antibodies were used at between 1/800 (S22) or 1/1000 (fibrin and muramidase), swine anti-rabbit IgG at 1:80 and PAP at 1:200. All sections were counterstained with haematoxylin. Sections of the nodules were also stained for ferric iron by Perls' reaction and with conventional trichrome and elastic tissue methods.

On 13 occasions the specificity of the staining reaction for fibrin (4 patients), IgG (4 patients), IgM (3 patients) and IgA (2 patients) was investigated by a blocking technique. In this method parallel batches of sections are stained:

1. In the usual way with the primary rabbit antibody, for example a 1/200 dilution of rabbit anti-human IgG.

2. With a primary antibody reagent made up of equal parts of a dilute rabbit anti-human IgG (1/200) and a concentrated goat anti-human IgG (1/5).

By virtue of its higher concentration the goat antibody binds preferentially to tissue antigens but cannot subsequently react with swine anti-rabbit labelled immunoglobulin. A marked reduction in staining is therefore anticipated when a mixture of antibodies is used. If this is not achieved the specificity of the reaction is doubtful. Absorption methods were used to assess the specificity of staining for ferritin and alpha-1 anti-trypsin (Blake et al. 1984).

Previous studies from Southampton have shown that the PAP technique employed in this investigation is a sensitive and reproducible method of demonstrating immunoglobulins in cells (Isaacson et al. 1980). Furthermore studies of renal biopsy material show that both the presence and distribution of immunoglobulin and complement components can be demonstrated in fixed tissue with great sensitivity using this method (MacIver et al. 1979). Comparative studies clearly demonstrate that serum proteins can be visualised with equal or greater efficiency, in comparison with immunofluorescence methods (Denk et al. 1977; Sinclair et al. 1981). The improved morphology obtained with formalin fixed paraffin embedded tissues also gives more accurate localisation when compared with frozen sections (MacIver and Mepham 1982). Data relating to the overall reliability of various markers for the identification of cells of the macrophage monocyte lineage has been previously published (Isaacson and Jones 1983; Isaacson et al. 1983).

Electron microscopy and X-ray microanalysis. Representative portions of biopsy nodules were cut into 1 mm^3 fragments and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH = 7.4) followed by 2% aqueous osmium tetroxide. All specimens were embedded in Spurr's resin following dehydration in ethanol. For morphological studies ultrathin sections mounted on copper grids were contrasted with lead citrate and uranyl acetate and viewed in a Siemen's Elimskop 102 electron microscope at an accelerating voltage of 80 Kv. Thicker sections (0.5 μm) also mounted on copper grids were carbon coated and analysed for the presence of iron using a Jeol JEM – 1200 EX electron microscope with computerized x-ray microanalytical facilities (Morris et al. 1984).

Results

1. Histological findings

Eighteen of the 20 specimens had the microscopic appearances of rheumatoid nodules, whereas two were bursae. The nodules showed a continuum of histological changes but in the majority there were multiple cavitating areas of confluent necrosis with surrounding pallisades of mononuclear cells (Fig. 1). In five nodules the appearances were less typical. The mononuclear infiltrates were less dense and pallisading poorly developed. In addition, central collagenous necrosis was less advanced (Fig. 2). These changes probably represent an early stage in the development of nodules. In contrast, in three otherwise typical nodules there were dense scar-like masses of poorly cellular collagen (Fig. 3), presumably the end stage in the evolution of a nodule.

The majority of blood vessels in and around nodules were entirely normal although a small proportion showed reactive intimal thickening. Occasional small arteries and veins were surrounded by lymphocytes. One vessel in the centre of a granuloma (Fig. 1) showed advanced necrosis with associated inflammatory cell infiltration. In a separate lesion a 300 μm diameter vein adjacent to several granulomata showed undoubted inflammatory vasculitis.

For practical purposes, Perls' stain for ferric iron was entirely negative.

2. Immunohistochemical results

Antisera to plasma proteins. As in all collagen rich tissues, non-specific background staining was frequently observed, particularly with antibodies to

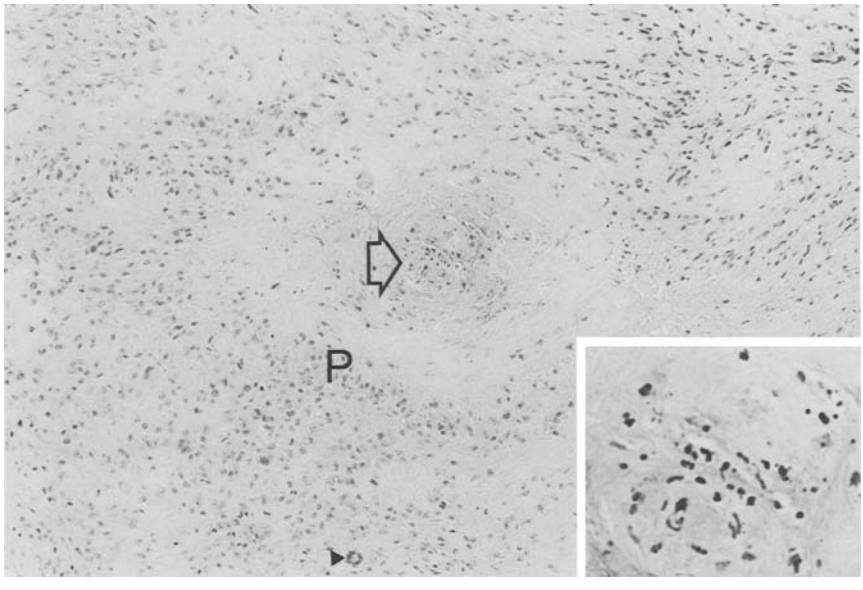

Fig. 1. Typical rheumatoid nodule with a necrotic vessel (arrow and inset) in the centre of a lesion. Note pallisades of macrophages, *P* and a giant cell (*arrowhead*). Haematoxylin and eosin ×110, inset ×270

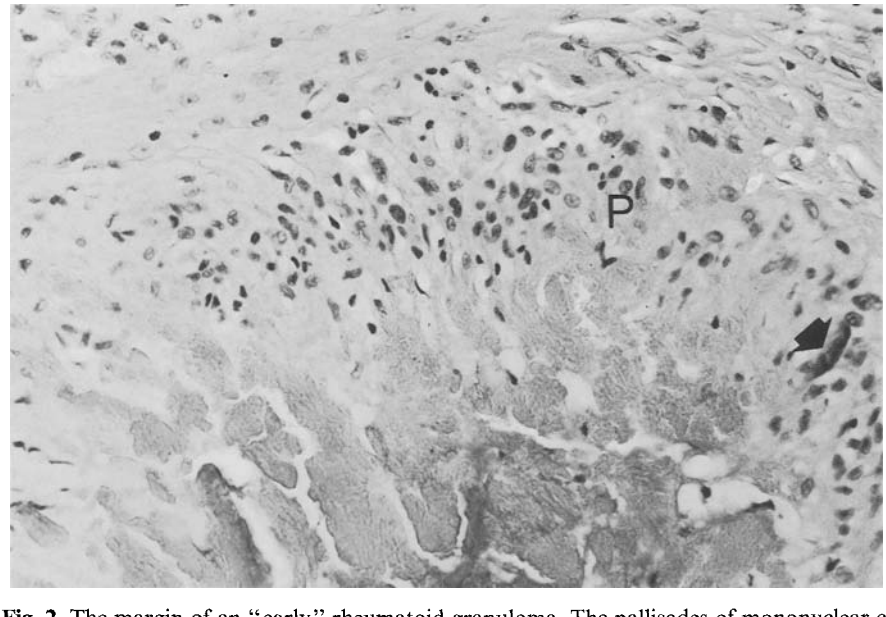

Fig. 2. The margin of an "early" rheumatoid granuloma. The pallisades of mononuclear cells, *P*, include a multi-nucleated form (*arrow*). The central core of collagen is necrotic but not as structureless as in more typical nodules. Haematoxylin and eosin ×250

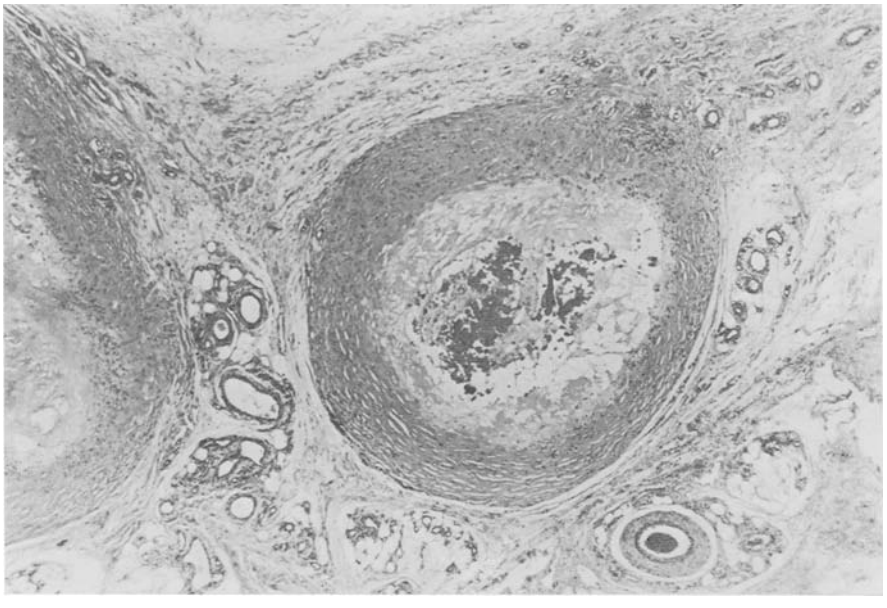

Fig. 3. A healing rheumatoid nodule surrounded by loose connective tissues containing small blood vessels. Part of another nodule is present at the left. Haematoxylin and eosin × 30

Table 1. Density of extracellular peroxidase staining reaction in central necrotic areas of rheumatoid granulomas using antisera to plasma proteins

	Albumin	Fibrin	IgG	IgM	IgA
Staining reaction					
Nil	0	0	3	7	9
Weak or focal staining	6	7	7	5	4
Strong staining	9	8	5	3	2

Figures refer to number of cases showing each type of reaction. Three cases in which immunohistochemical staining was technically unsatisfactory or blocking techniques imperfect are excluded

albumin and IgG. Dilution of the primary antibody reduced and sometimes eliminated background staining and this was usually accompanied by a reduction in seemingly genuine staining within plasma cells and macrophages, or in the centre of granulomatous lesions.

As shown in Table 1, extracellular staining for albumin and fibrin was present in all nodules, usually as confluent deposits occupying the entire central area of necrosis and encroaching into the surrounding pallisades of mononuclear cells (Fig. 4).

In contrast, staining with immunoglobulin antibodies was more patchy (Fig. 5a). Typically, deposits were finely granular and were present at the

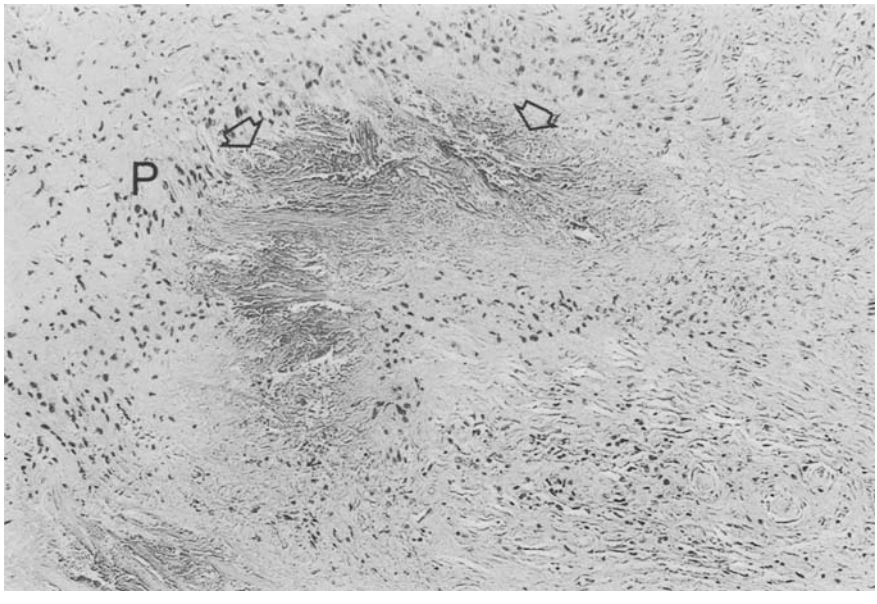

Fig. 4. PAP immunoperoxidase stain for fibrin. Note strong staining reaction (*arrows*) in necrotic centre of granuloma. Pallisading macrophages, *P*. ×75

periphery of areas of necrosis (Fig. 5b). Generally, there was no evidence of immunoglobulin deposition in blood vessel walls although a fine rim of staining was often present at the intimal surface, presumably deposited from plasma protein. Transferrin antibodies gave a similar reaction in blood vessels. Patchy deposits of IgG were detected in the small vein which showed vasculitis but stains for IgM, IgA and complement were negative in this vessel. Strong intracellular staining for IgG, IgM and occasionally IgA was observed in the cytoplasm of plasma cells, but these were rarely numerous and usually confined to the periphery of nodules. Light diffuse cytoplasmic staining for albumin, IgG and IgM was present in a small proportion of the monoclear cells forming pallisades around areas of necrosis. Occasionally the immunoglobulin reaction product had a finely granular appearance.

No significant complement staining was identified. Both kappa and lambda light chains were present in the cytoplasm of plasma cells but no other staining was observed when these antibodies were used.

Staining reactions of mononuclear cells. There was considerable variation in the intensity of staining of mononuclear cells arranged around areas of necrosis. At one end of the spectrum an intense granular cytoplasmic staining was observed in virtually all pallisading cells with antibodies to apoferritin (Fig. 6). On the otherhand, very little reaction was obtained with antibodies to alpha-1 anti-trypsin and transferrin. Intermediate results were noted with the polyclonal rabbit antibody raised against highly purified preparations of human monocytes (S22) and muramidase antibodies. Again the pattern was granular and this granularity was invariably more marked with S22 (Fig. 7).

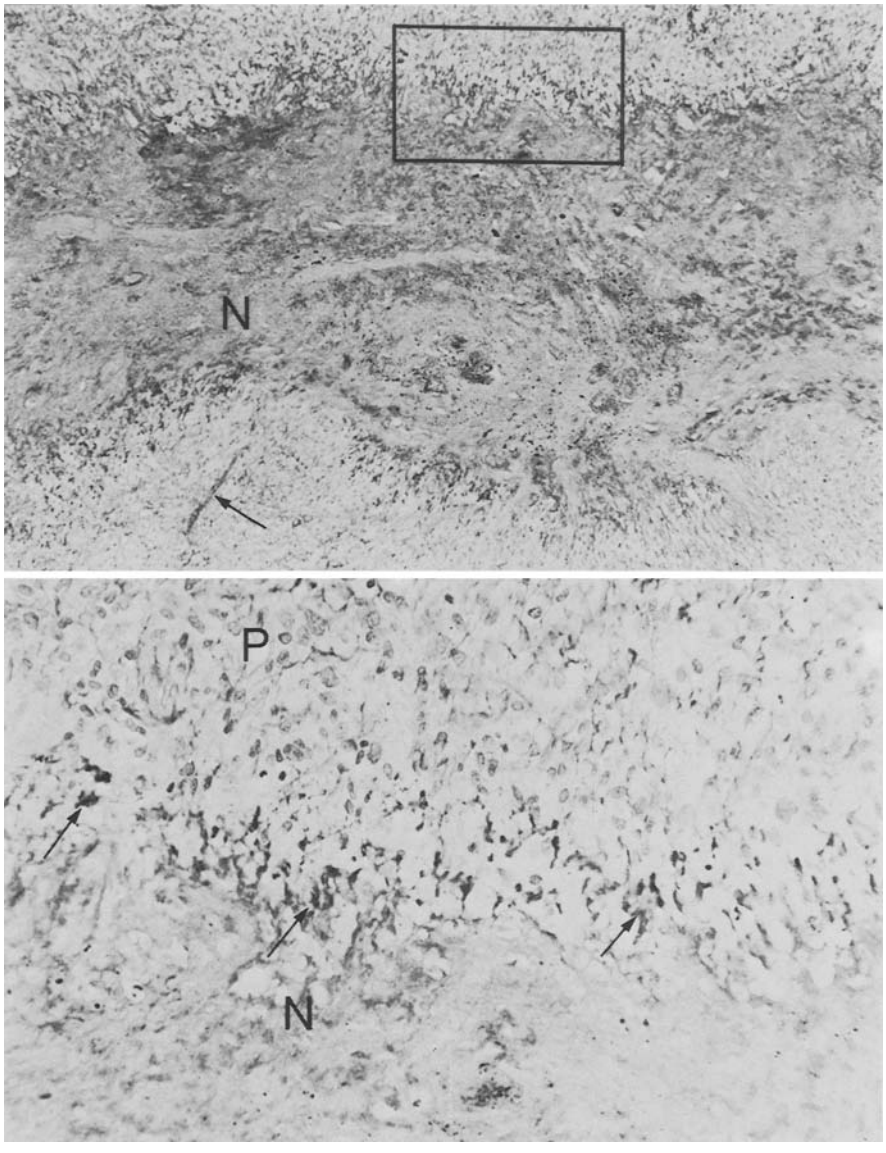

Fig. 5a. Indirect immunoperoxidase stain for IgG. There are patchy deposits of immunoglobulin in the necrotic centre of the granuloma (*N*). A small vessel containing IgG in its lumen is arrowed. × 80. A magnified view of the boxed area is shown in **b**. **b** The rim of the granuloma with pallisading macrophages (*P*) above and necrosis (*N*) below. Note the granular deposits of IgG at the margin of the necrotic area (*arrows*). × 375

3. Ultrastructure and X-ray microanalysis

Nodules consisted of collections of mononuclear macrophage-like cells interspersed with collagen and fibrin (Fig. 8). All mononuclear cells contained collections of rounded bodies with granular material of varying electron

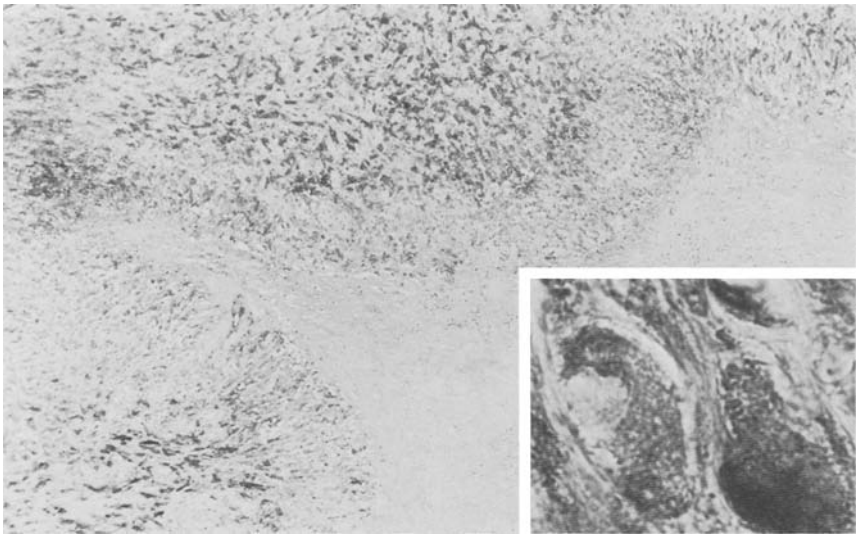

Fig. 6. Indirect immunoperoxidase stain for apoferritin around the margins of a necrotic granuloma. ×60. *Inset:* there is intense granular staining for ferritin in the cytoplasm of mononuclear cells. ×1150

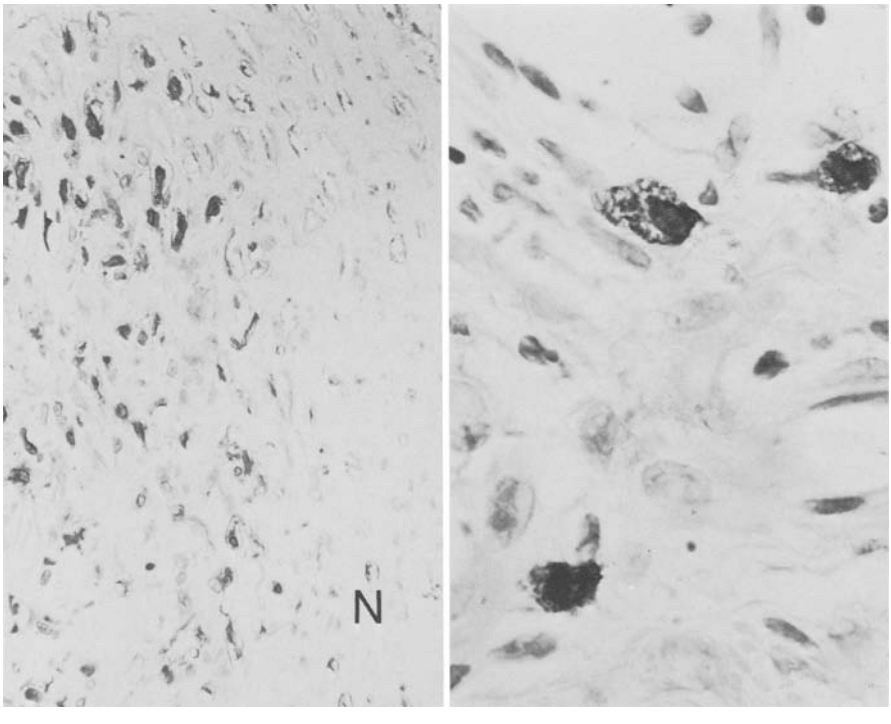

Fig. 7. PAP immunoperoxidase stain for S22 (polyclonal monocyte antibody). *Left.* Many of the cells surrounding the necrotic centre of the granuloma (*N*) give a positive reaction. ×150. *Right.* The pattern of granular cytoplasmic staining. ×600

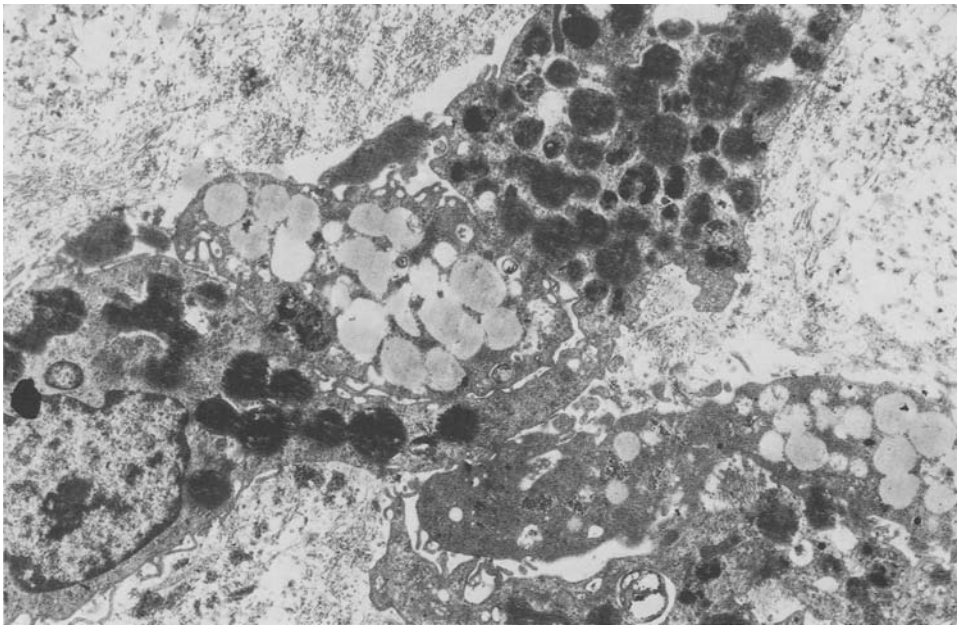

Fig. 8. Electron micrograph of a typical rhematoid nodule. These are collections of macrophage-like mononuclear cells containing rounded inclusions of varying electron density. Cells are surrounded by a matrix of collagen and some fibrin. × 6000 (approx)

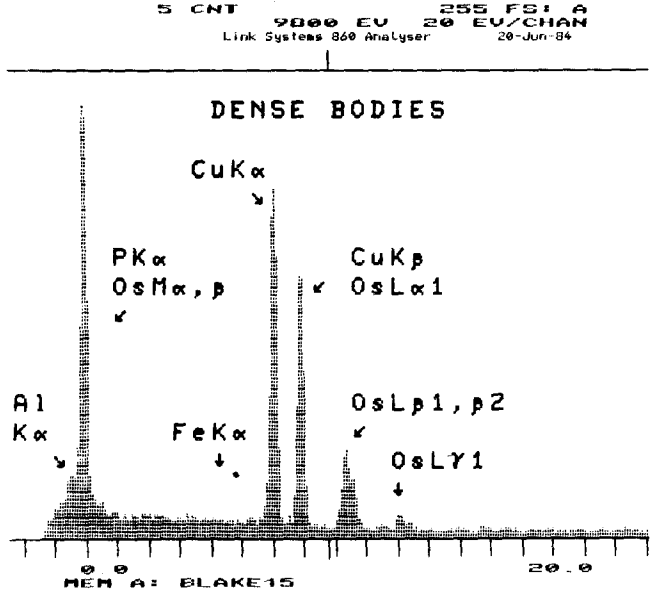

Fig. 9. Electron probe x-ray microanalysis computer print-out of an inclusion body (see Fig. 8). Note absence of a peak for iron (FeKα). The copper peaks (CuKα and CuKβ) are derived from copper grids supporting the specimen and the osmium peaks (OsMα,β, OsLα1, β1, β2 and OsLγ1) from the fixation procedure. The height and range of osmium peaks present indicates a high level of binding which produces the electron density seen in Fig. 8

density. These bodies were membrane bound and morphologically comparable to classical siderosomes. Surprisingly, therefore, x-ray microanalysis of such bodies (Fig. 9) shows that they are entirely free of iron, electron density being due to the very high level of osmium binding following fixation.

Discussion

The results of this study indicate that vasculitis is uncommon in established rheumatoid nodules. Plasma proteins were identified in the necrotic centres of nodules but in amounts in approximate proportions to their concentration in the plasma. There was no evidence to suggest that immunoglobulin or complement was preferentially deposited in, or around, nodules or in the walls of arteries. They appear to have leaked non-specifically into areas of necrosis.

Necrosis is a consistent feature of the rheumatoid granuloma and is even present in nodules that have been present clinically for less than 7 days (Rasker and Kuipers 1983). Both the present study, and an earlier Japanese report (Fukase et al. 1980), suggest that the histological appearances progress with time and that necrosis with cavitation is followed by healing with fibrosis. It is a paradox that in rheumatoid disease small necrotising digital and rectal lesions (Scott et al. 1981) should be so strongly associated with vasculitis yet comparatively large nodules with extensive necrosis should have no obvious vascular basis. It is possible that an episode of subcutaneous vasculitis initiates the necrosis but by the time of biopsy the histological signs of vasculitis have subsided, or perhaps as shown in Fig. 1, the relevant vessels have themselves undergone necrosis. Nevertheless, a recent study of 13 nodules biopsied only a matter of days after their first appearance also showed a low incidence of vasculities (Rasker and Kuipers 1983). In 5 of the 13 cases deposits of immunoglobulin or complement were identified in vessels by an immunofluorescent technique, usually in cases in which vasculitis was present in corresponding conventional sections.

Immunofluorescent methods have inherent disadvantages. Frozen sections must be used, the reaction product is unstable and as the preparations must be examined with a fluorescent microscope precise localisation of antigen can be difficult. In contrast, the immunoperoxidase methods we use allow a variety of antigens and antibodies to be accurately and sensitively identified in permanent counterstained preparations. Despite the differences in these methods it is interesting that a separate immunofluorescent study has also reported plasma protein deposits in the necrotic centres of rheumatoid nodules (Fukase et al. 1980). With a single exception our techniques failed to demonstrate either immunoglobulin or complement around, or in, blood vessels. We, therefore, have no direct evidence to support the view that immune complex deposition is the primary event in rheumatoid nodules.

Our view is more that rheumatoid nodules are chronic inflammatory lesions in which mononuclear cell infiltration and necrosis are the chief histological features. It was of particular interest that such strong reactions were obtained in mononuclear cells with antibodies to ferritin and purified

human macrophages. In contrast, reactions with muramidase were only weak and alpha-1 anti-trypsin virtually negative. Although we obtained rather similar results in a study of synovium (Blake et al. 1984) the lack of alpha-1 anti-trypsin staining is rather puzzling. This antigen is readily expressed by both reactive and neoplastic macrophages, particularly Kupfer cells and pulmonary alveolar macrophages (Isaacson et al. 1981). The strong staining in Kupfer cells can be ascribed to direct synthesis and in pulmonary macrophages antiproteases are presumably reabsorbed from the alveoli. There is no evidence to suggest that macrophages in rheumatoid lesions synthesize antitrypsins but it is surprising that they do not accumulate it in much the same way as alveolar macrophages. It is clear that the mononuclear phagocyte populations present in chronic inflammatory lesions represent a heterogenous group of cells, some recently recruited, others representing mature tissue histiocytes (Ryan and Spector 1970), and all showing varying degrees of activation (Davis and Allison 1976). The demonstration of cytoplasmic material will, therefore, depend on the balance of synthesis and export in these cells and it is, therefore, not surprising that individual cells may vary in their phenotype, as shown by immunostaining.

It is equally intriguing that pallisading macrophages should contain so much ferritin yet Perl's stain for iron be entirely negative. Indeed, in a quite separate study in the Southampton laboratories very similar results were observed in macrophages in reactive and neoplastic lymph nodes (Jones et al. 1984). We have shown that Perl's staining of gelatin embedded ferritin samples of known iron loading is negative at <900 mols Fe/mol protein. It is possible, therefore, that substantial amounts of ferritin iron could be present in the absence of Perl's staining. X-ray microanalysis, however, shows that in the nodule macrophages iron is *totally* lacking and that the ferritin demonstrated immunohistochemically is therefore pure apoferritin.

These observations support the view, first expressed by Hershko and Konijn (1977) that the initial stimulus to apoferritin production is not incoming iron but a mediator generated by the inflammatory process. It is likely that this mediator is interleukin I (Lee 1983) generated by many cell lines including macrophages and acting on the reticulo-endothelial system to induce an acute phase response. One might suggest that a common feature of rheumatoid disease is its predilection to involve organs and structures subject to sheer stress. The joint is a clear example as are the pleural and pericardial surfaces. The sites of nodules at pressure points in the subcutaneous tissue and their occasional presence at other sites of sheer stress suggest that trauma may induce an exaggererated tissue response with over production of interleukin I, possibly from fibroblasts. The macrophage response in terms of apoferritin production would be appropriate, but their basic metabolism in terms of muramidase and alpha-1 anti-trypsin production is not. These findings suggest that kinetic studies of the generation and turnover of antiproteases, interleukin 1 and macrophage lysosomal enzymes in rheumatoid tissues may be considerable interest.

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