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Antigen-induced arthritis in the rabbit: ultrastructural changes at the chondrosynovial junction

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Summary. Structural changes at the chondrosynovial junction of the lateral border of the lateral femoral condyle have been studied by electron microscopy in rabbits with antigen-induced arthritis of 6 h–27 days duration. Rapid changes in the collagen fibrils of the extracellular matrix in the synovial lining and articular cartilage were noted. Collagen fibrils with unusually large diameters were observed. Overgrowth of cartilage by inflamed synovium was seen within 3–6 days of induction of arthritis and by day 12 the interface between these two tissues was largely indistinguishable. The synovial pannus at this time was fibrotic and infiltrated with plasma cells, lymphocytes and macrophages. Few polymorphonuclear leucocytes were found in the developing pannus. Macrophages were found with extended processes which enveloped neighbouring cells. Some blood vessels had thickened endothelial cells though lymphocytes were not observed in their vicinity. This study reveals the rapidity with which synovial pannus can develop and suggests that there are a number of mechanisms operating to cause cartilage breakdown in antigen-induced arthritis.

Keywords: antigen-induced arthritis, rabbit, synovium, cartilage, ultrastructure

Rheumatoid arthritis (RA) is one of the commonest causes of disability in developed countries, affecting approximately 1% of the population. Disability is due to chronic inflammation in the synovial lining leading to destruction and remodelling of cartilage, bone, tendon and ligament (Henderson & Edwards 1987). Such progressive damage occurs primarily at sites where the inflamed synovial lining meets and overgrows cartilage, an area known as pannus. This leads to the centripetal erosion of these tissues during the course of the disease. A detailed description of the develoment of this tissue is necessary for an understanding of the processes involved in tissue destruction in RA. A number of histological and electron microscope studies of pannus have revealed that synovial pannus in RA is a cellularly diverse tissue (Kobayashi & Ziff 1975; Barrie 1981; Fassbender 1983; Bromley & Woolley 1984). This diversity is presumed to be due to the range of samples available to the investigators. These samples would have come from

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a range of joints and from patients with a range of disease durations. By the nature of the disease it is also difficult to follow the progress of pannus progression.

The study of pannus development may be readily undertaken in animal models of RA. Of the available experimental models in animals, antigen-induced arthritis in the rabbit (AIAR) comes closest to the human disease in terms of cellular infiltrate and architectural changes in the tissue matrix (Dumonde & Glynn 1962; Edwards *et al.* 1988). Alterations in synovial lining metabolism also closely resemble those in human tissue (Henderson & Glynn 1981; Henderson & Pettipher 1985). The sequence of biochemical events occurring in the synovial lining and articular cartilage during the development of AIAR has recently been studied in detail (Henderson & Glynn 1981; Henderson 1981, 1983, 1984; Henderson & Higgs 1987; Pettifer & Henderson 1988; Pettipher *et al.* 1988, 1989a) and has been related to morphometric changes in the cellular infiltrate using light microscopy (Edwards *et al.* 1988; Pettiphor *et al.* 1989b). However, the ultrastructure changes associated with these biochemical and light microscope findings have not been described in detail.

The aims of the present study were to establish to what extent ultrastructure confirms the similarities in cellular changes between the rabbit lesion and the human disease during the development of AIAR and to gain more detailed information on the ultrastructural changes occurring during the development of pannus. The chondrosynovial junction at the lateral border of the lateral femoral condyle was chosen as the area to study pannus development. In this region the synovium overlying the periosteum of the condylar bone meets the articular hyaline cartilage and, during inflammation, encroaches over the cartilage to form pannus. This area is therefore appropriate for following the development of synovial pannus and its relationship to bone and cartilage damage.

Materials and methods

Animals

Mature New Zealand White male rabbits, weighing 3–4 kg, were used in all experiments.

Induction of arthritis

Animals were sensitized with 4 mg ovalbumin (Sigma) in 1 ml Freund's complete adjuvant (Gibco) in multiple intradermal sites in the suprascapular region. Animals were resensitized 14 days later using the same protocol. Five days after the second resensitization arthritis was induced in the right knee joint by injecting 1 ml of a sterile solution of ovalbumin (5 mg/ml) into the joint cavity. The contralateral joint was injected with sterile saline to act as a 'within-animal' control. Animals were killed at intervals from 6 h to 27 days following intra-articular challenge.

Tissue preparation

At the various intervals after challenge, the animals were killed by intravenous injection of Nembutal. The challenged knee joints were exposed and wedges of tissue from the chondrosynovial junction of the lateral aspect of the lateral femoral condyle were obtained with a sharp scalpel. The contralateral knee was sampled from representative animals in each experimental group to provide control tissue. The specimens were immediately fixed in 2.5% glutaraldehyde (phosphate buffer, pH 7.2) at room temperature for at least 2 h, followed by post-fixation for 1 h in 1% aqueous osmium tetroxide. Fixed specimens were then rinsed in distilled water, dehydrated in an ascending ethanol series, cleared in propylene oxide, and infiltrated with increasing concentrations of Araldite. Polymerization in pure Araldite took place over 48 h at 60°C. Semi-thin sections were obtained with glass knives, stained in toluidine blue and assessed under light microscopy for suitability. When useful

regions of the specimens were identified, thin sections were cut on a diamond knife and picked up on uncoated copper grids. The sections were stained with 2% aqueous uranyl acetate followed by 1% Reynold's lead citrate. Sections were observed with a JEOL 1200 EX transmission electron microscope at 80 kV and micrographs obtained at magnifications of ×4000–20000.

Quantitative morphometry

Based on planimetric principles (Weibel 1969), the assessment of collagen fibril diameters was determined using a Weibel multipurpose point counting grid with 1 cm point spacing laid over randomly selected micrographs from each set of experimental and control joints. Collagen fibrils sampled in predetermined grid squares were counted and measured if they appeared circular in cross-section. To minimize random sampling errors samples in each group of animals were pooled ($n>400$) for both experimental and control tissues.

Results

Semi-thin resin sections stained with toluidine blue confirmed the presence of synovial tissue with a surface layer of lining cells and underlying fatty or areolar matrix adjacent to and merging with hyaline cartilage. Progressive development of a typical pannus overlying and replacing cartilage and undercutting erosive granulation tissue resembled that reported in RA (Henderson & Edwards 1987). In all control material examined there were no observable deviations from the known normal ultrastructure. For presentation of results, five groups were chosen corresponding to animals killed after intra-articular challenge: 6 h–1 day, 3–6 days, 12–13 days, 20 days, and 26–27 days.

Group I (6 h–1 day; four animals)

Within 1 day, while the cells within the synovial lining appeared normal, the extracellular matrix of the synovium was noticeably disrupted. Instead of a regular meshwork of loosely bundled collagen fibrils there were extensive regions where collagen fibrils were depleted. Large deposits of fibrinous material were found and closely associated among these deposits were some apparently normal appearing collagen fibrils. Within the deep synovial tissue there were oedematous regions containing some polymorphonuclear leucocytes, cellular debris, few collagen fibrils, and remnants of necrotic cells (Fig. 1). The outlines of the deeper-lying synovial cells were thrown into extensive cytoplasmic folds. Variable numbers of blood vessels were found among the specimens studied. In many the lumen was distended with amorphous deposits and red blood cells suggesting intravascular coagulation. The endothelial cells appeared normal.

There were no obvious signs of damage to the surface of the cartilage which was covered with an amorphous, lightly stained deposit of variable thickness (Fig. 2). An interesting observation was the occurrence of clumps of unusually large-diameter collagen fibrils in random orientation within the cartilage matrix (Fig. 3). These collagen fibrils had diameters as large as 1000–2000 Å which are very much beyond the normal spread of cartilage collagen fibril diameters. They were found only near the surface of the cartilage next to clumps of cell debris, which were considered to be remnants of necrotic chondrocytes.

Group II (3–6 days; six animals)

By this time the synovium had begun to encroach over the adjoining cartilage. The surfaces of the overlying synovium and the cartilage became obscured owing to the close apposition of the synovial lining onto the cartilage. Macrophages could be seen near to the cartilage surface. These phagocytic cells contained dense and intracytoplasmic material which could presumably be ingested matrix material or siderosomes. Many necrotic cells, possibly arising from the

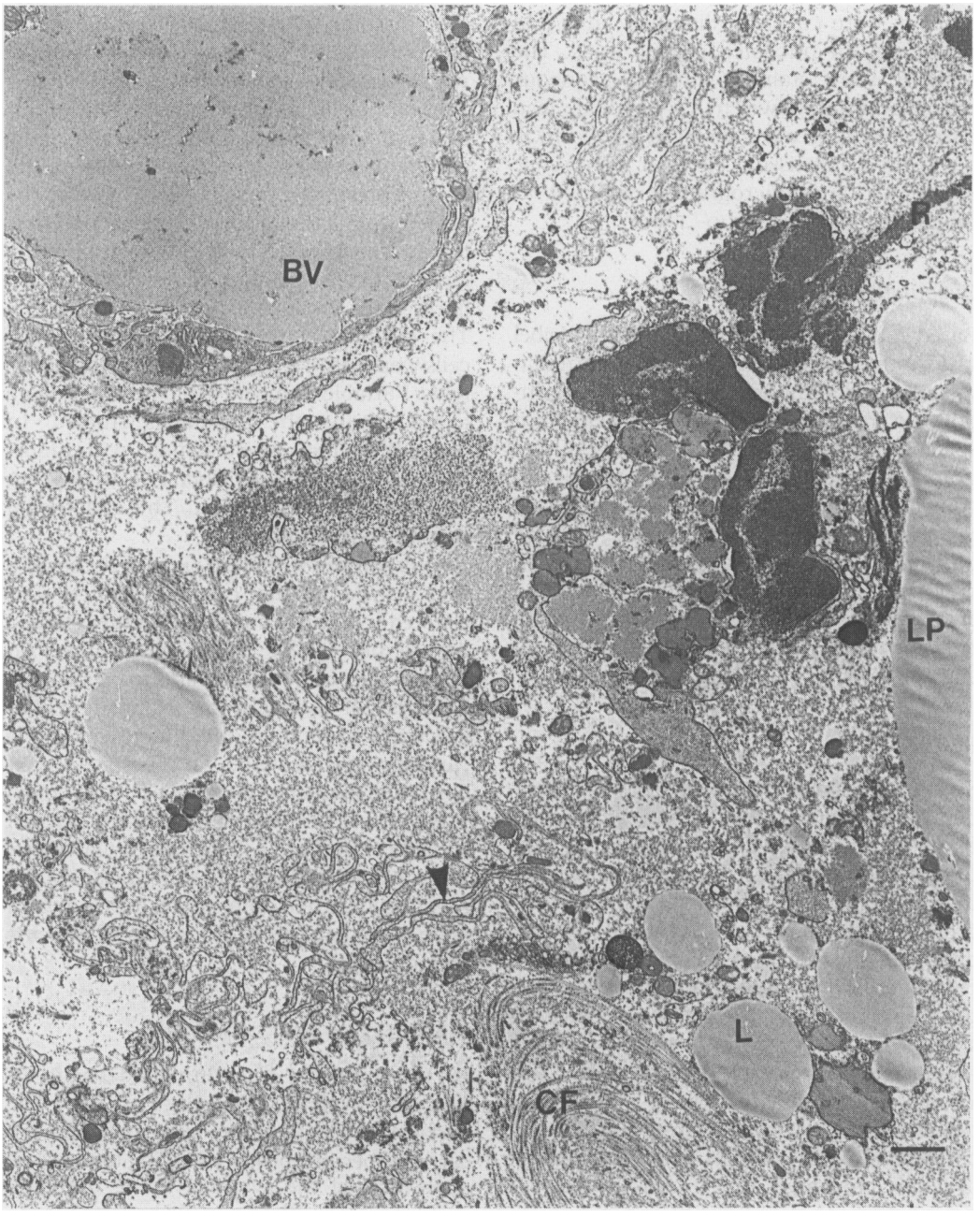

Fig. 1. 1 day: Deep synovial tissue. This region shows a few bundles of collagen fibrils (CF) among extensive cytoplasmic folds (arrows), lipid droplets (L), and cellular remnants (R). The blood vessel (BV) and lipocyte (LP) appear normal. Bar, 1 μm.

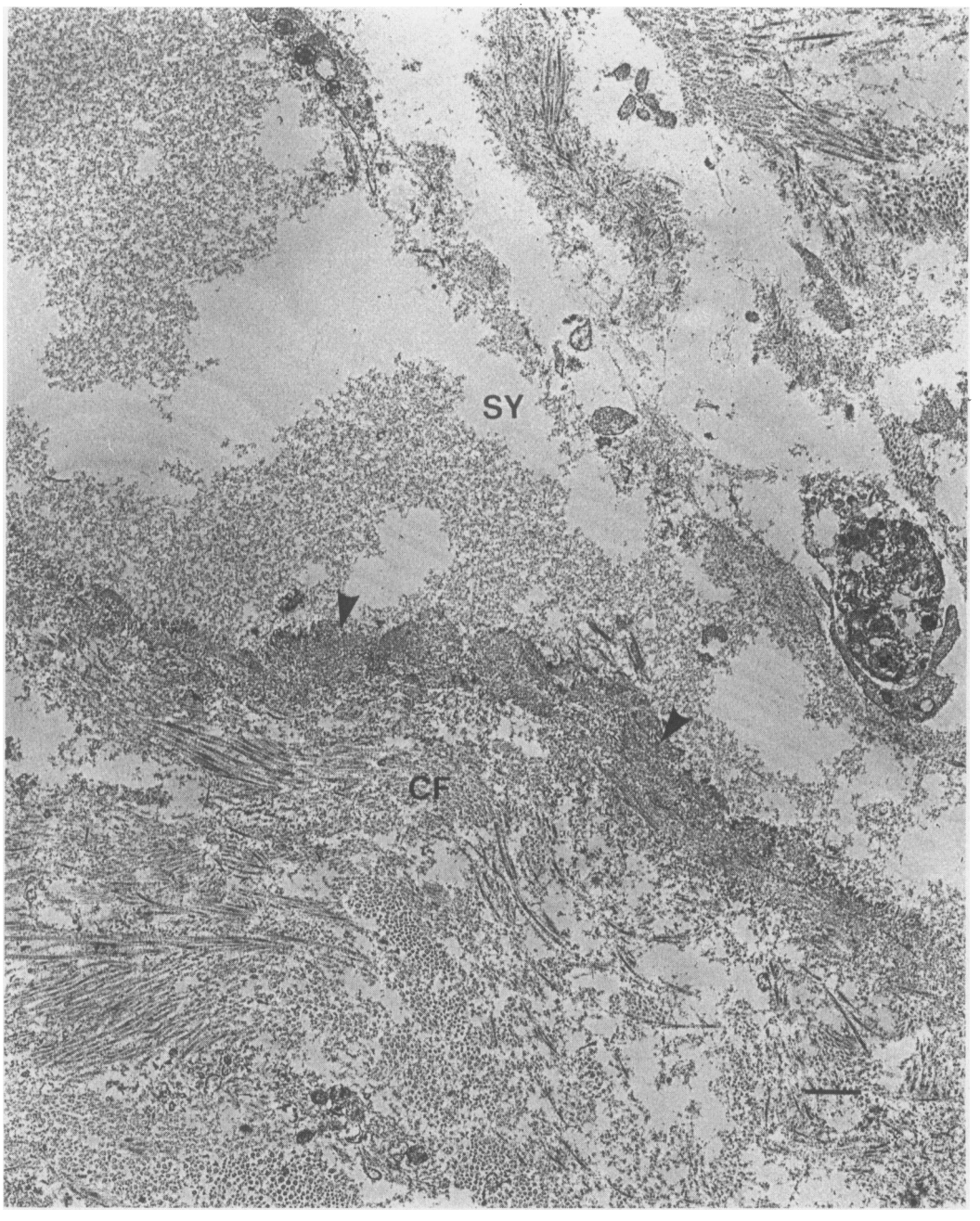

Fig. 2. 6 hours: The cartilage surface appears normal, with a layer of amorphous, fibrin-like material (arrows) covering a loose meshwork of collagen fibrils (CF). Collagen fibrils and synovial cells may be seen in the synovial cavity (SY). Bar, 1 μm.

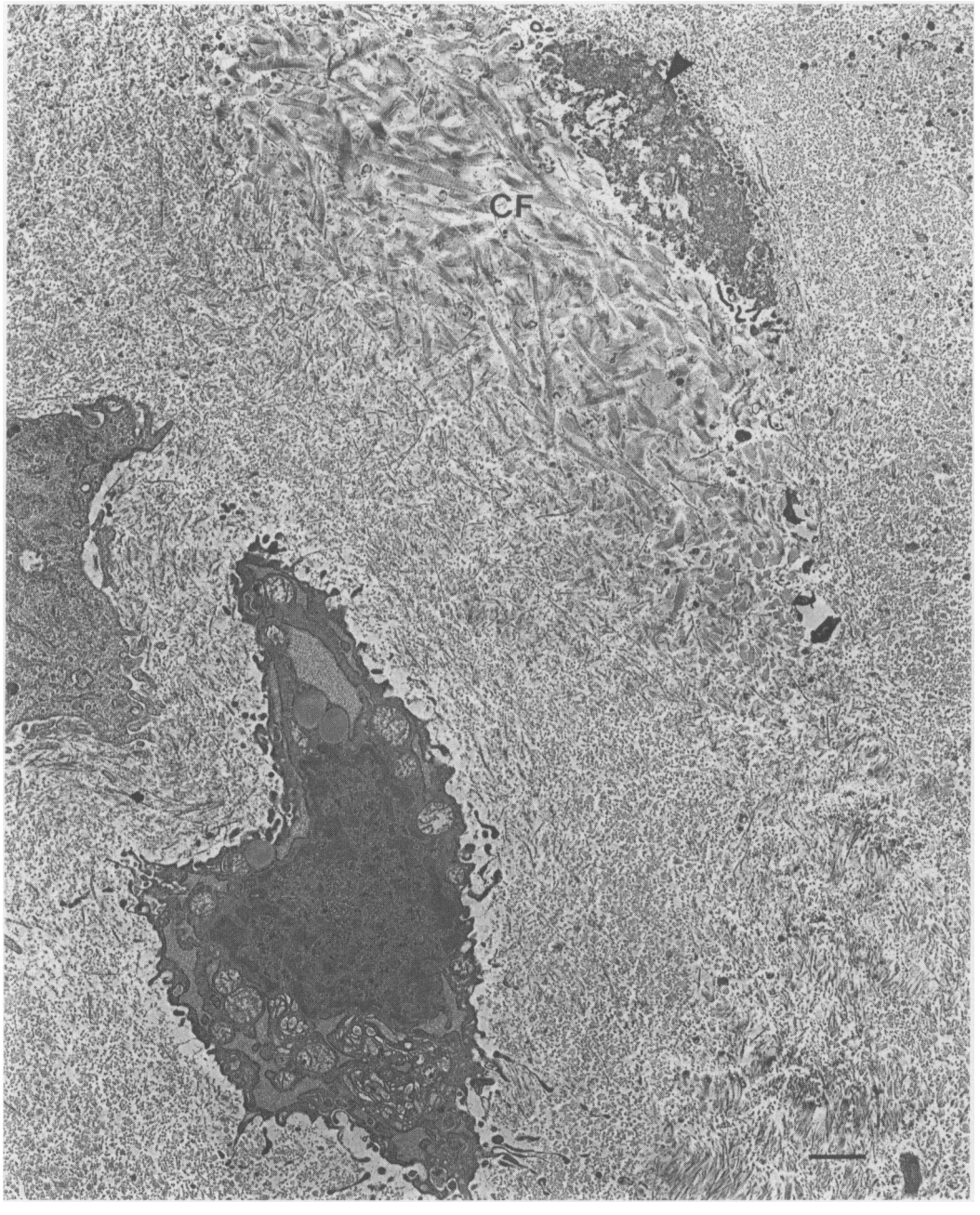

Fig. 3. 6 hours: The superficial region of cartilage showing a clump of collagen fibrils (CF) with large diameters close to a necrotic cell (arrow). Other chondrocytes appear healthy. Bar, 1 μm.

synovial lining or chondrocyte populations, could be seen. The matrix contained disrupted regions consisting of loose collagen bundles, lipid droplets, cell remnants and fibrin deposits.

Large aggregates containing fibrin, erythrocytes, collagen fibrils, a few polymorphs and necrotic cells could be observed within the deep synovial tissue. Some of the cells contained siderosomes suggesting phagocytosis of erythrocyte breakdown products.

There was no noticeable increase in the number of blood vessels, but among those that were present were a number with a congested lumen containing polymorphonuclear cells, lymphocytes and erythrocytes. A proportion of vessels had a thickened endothelium of pseudocolumnar appearance (Fig. 4). Vessels with this tall endothelial appearance were not associated with lymphocytes. There was also significant hypertrophy of vascular smooth muscle cells.

Erosion of the cartilage surface was noted. Extensive fibrin aggregates were found near and on the cartilage surface. Less densely stained than fibrin were globular masses of amorphous material (Fig. 5), suggesting either breakdown products of the collagen and proteoglycan matrix or material adsorbed onto a damaged surface. The material occurred amongst collagen fibrils as far as 15 μm deep into the cartilage, in a concentration gradient, densely packed near the surface and more dispersed deeper into the cartilage. The density of these deposits appeared to be inversely related to the amount of fibrillar collagen. Within the cartilage, localized areas of matrix degradation were usually associated with cellular remnants, necrotic chondrocytes and lipid droplets. As in Group I, a few clusters of very large collagen fibrils were found, some of which measured up to 7000 Å in diameter. Where granulation tissue overlay the bone directly, the fibrous matrix contained clumps of fibrin and collagen fibrils (Fig. 6). Osteoclasts and osteoblasts were often unrecognizable because of necrosis.

Group III (12–13 days; four animals)

By 12–13 days, the interface between synovium and cartilage had become unrecognizable. The whole section appeared highly fibrotic with a heavy infiltration of close-packed plasma cells, lymphocytes and macrophages. Macrophages were observed to extend processes which enveloped neighbouring cells, especially plasma cells (Fig. 7). Collagen fibrils were found within intracellular vacuoles in macrophages, and intracytoplasmic siderosomes were often observed (Fig. 8). Within the fibrotic matrix of the pannus were fibroblasts with extensive pseudopods. The diameter of collagen fibrils showed a broad frequency distribution, ranging from 176 to 995 Å ($n=800$). The majority of the fibrils fell within a normal Gaussian shaped distribution with a modal diameter of 250–300 Å. The rest were broadly distributed around the 650–750 Å mode. This 'bimodal' frequency distribution is clearly seen in Fig. 9. The collagen fibril diameters in control tissues were more narrowly distributed, with a range of 287–695 Å ($n=700$). The distribution profile was generally normal with the mode at 350–400 Å.

In areas of cartilage where the synovium had not encroached, the surface was covered with thick masses of inflammatory aggregates of fibrin, collagen fibrils, lipid droplets, and other cellular remnants. Within the deeper zones of the cartilage, many chondrocytes were found to be necrotic. Blood vessels had uncongested lumens and normal endothelium.

Group IV (20 days; three animals)

By 20 days the pannus increased in thickness to about 1 mm in some specimens. The matrix was highly fibrotic and densely packed with collagen fibrils, fibroblasts and lymphocytes. Most of the other observations from Group III animals were also found in this group, except that intracellular collagen fibrils were not found in any of the macro-

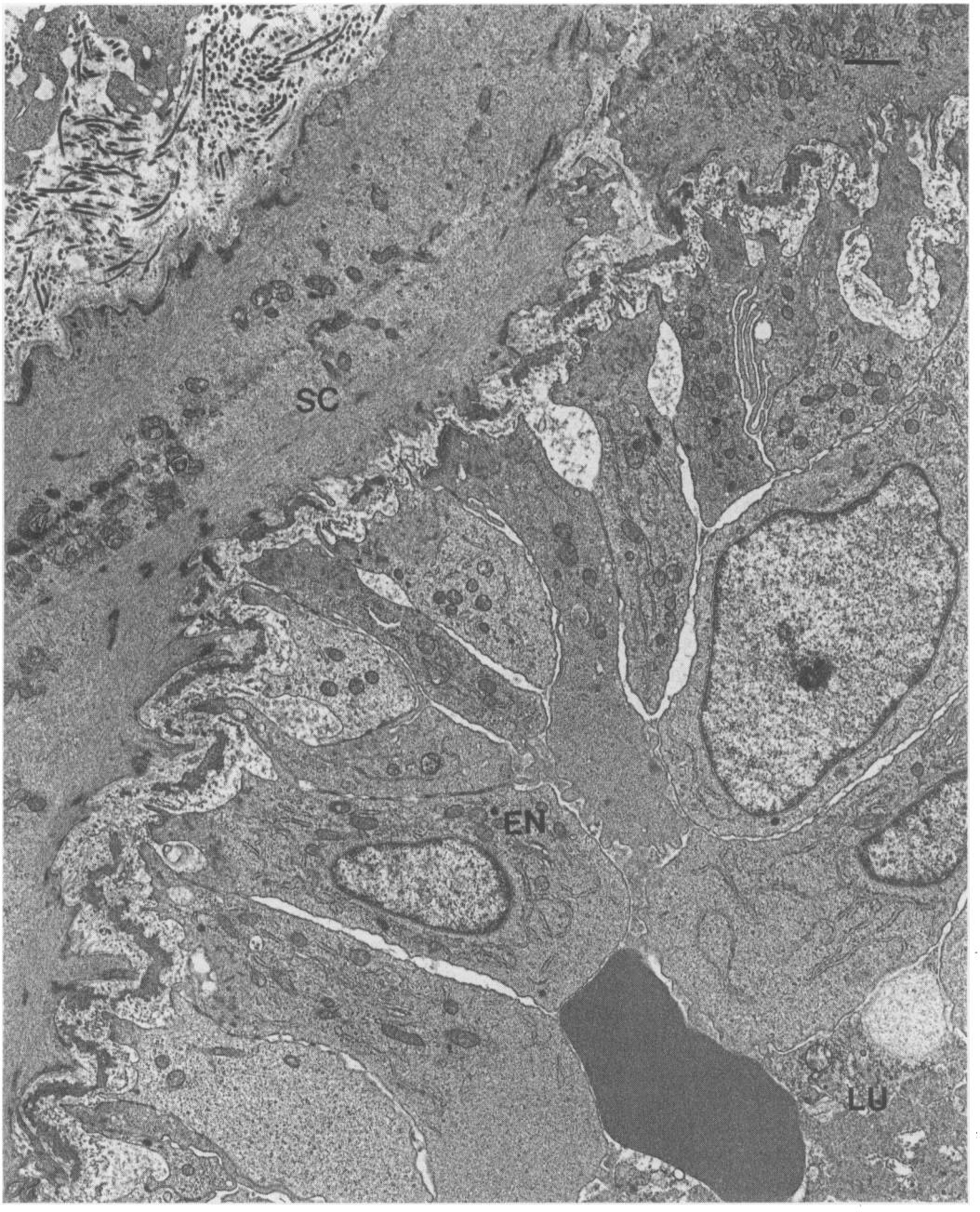

Fig. 4. Day 3: Synovial vessel. The endothelium (EN) is tall and the small lumen (LU) is congested. The smooth muscle cell (SC) is grossly enlarged. Bar, 1 μm.

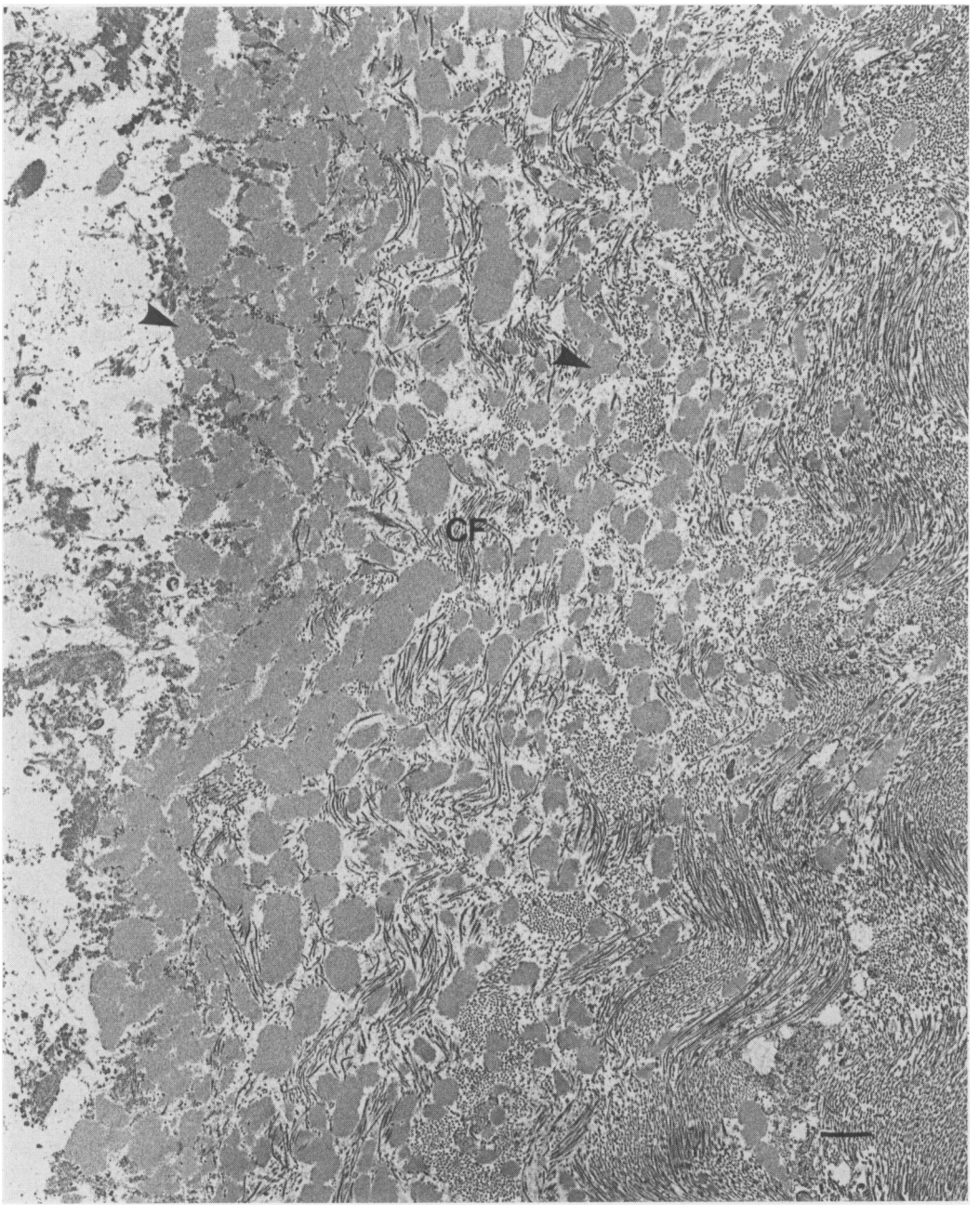

Fig. 5. Day 3: Cartilage surface. Amorphous deposits (arrows) can be seen amongst the cartilage collagen fibrils (CF); these are dense along the superficial layers and in decreasing amounts deeper into the cartilage. Bar, 1 μm.

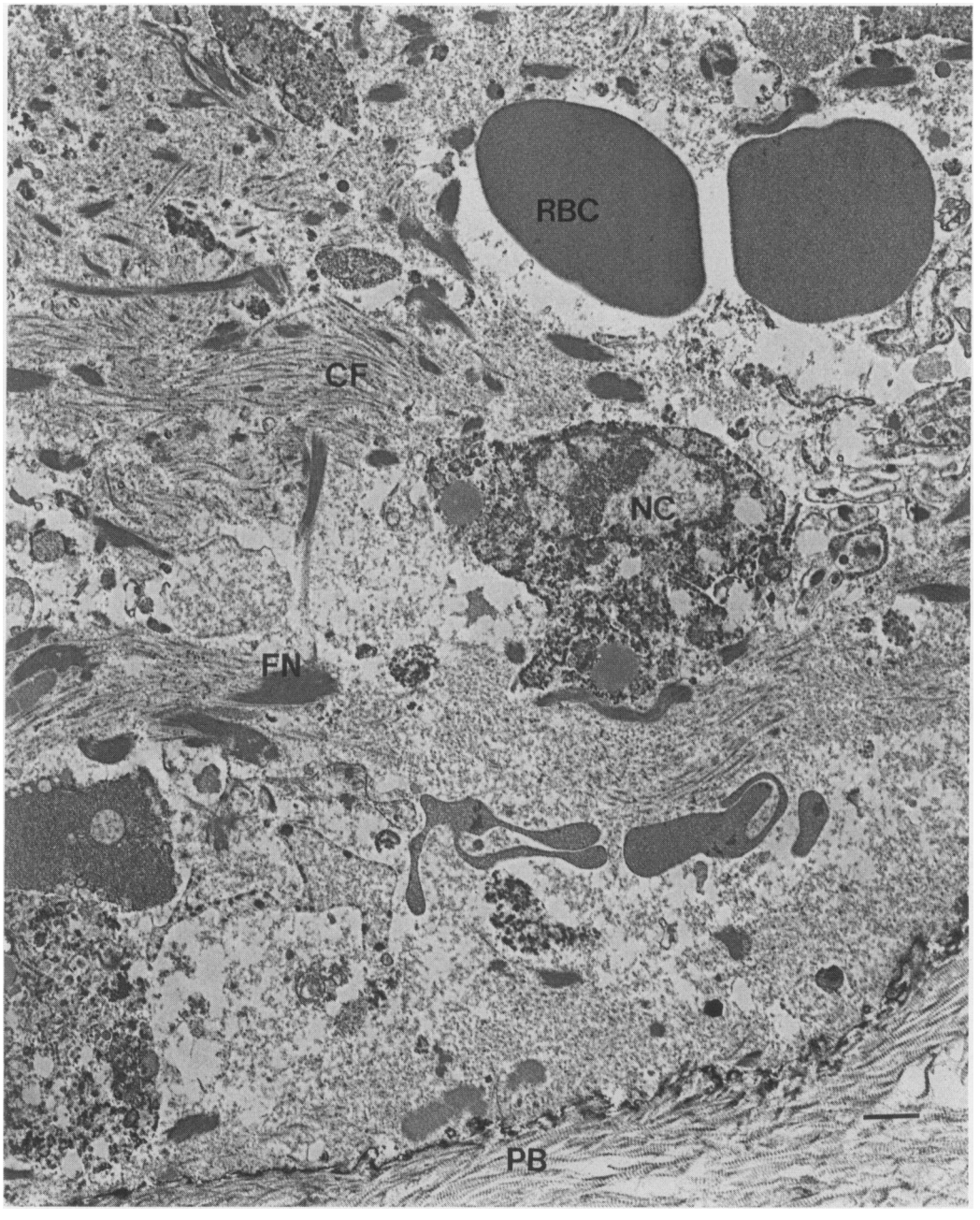

Fig. 6. Day 3: The perichondral bone (PB) is covered by the inflamed synovium, and the region contains an inflammatory infiltrate of fibrin (FN), necrotic cells (NC), erythrocytes (RBC), and some collagen bundles (CF). Bar, 1 μm.

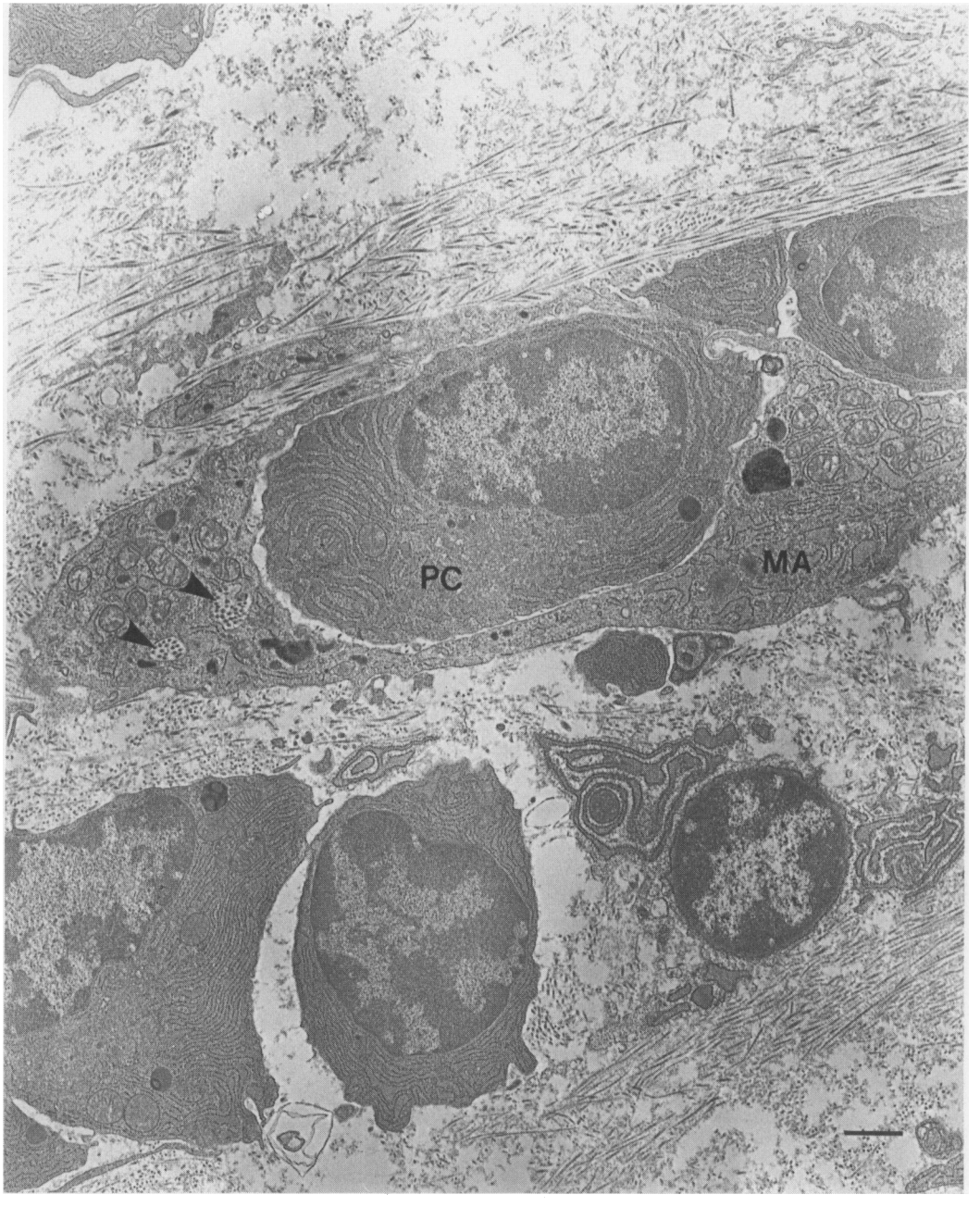

Fig. 7. Day 12: In this cell cluster within the synovium, a macrophage (MA) is observed enveloping a plasma cell (PC). It also contains phagocytotic vacuoles with collagen fibrils (arrows). Bar, 1 μm.

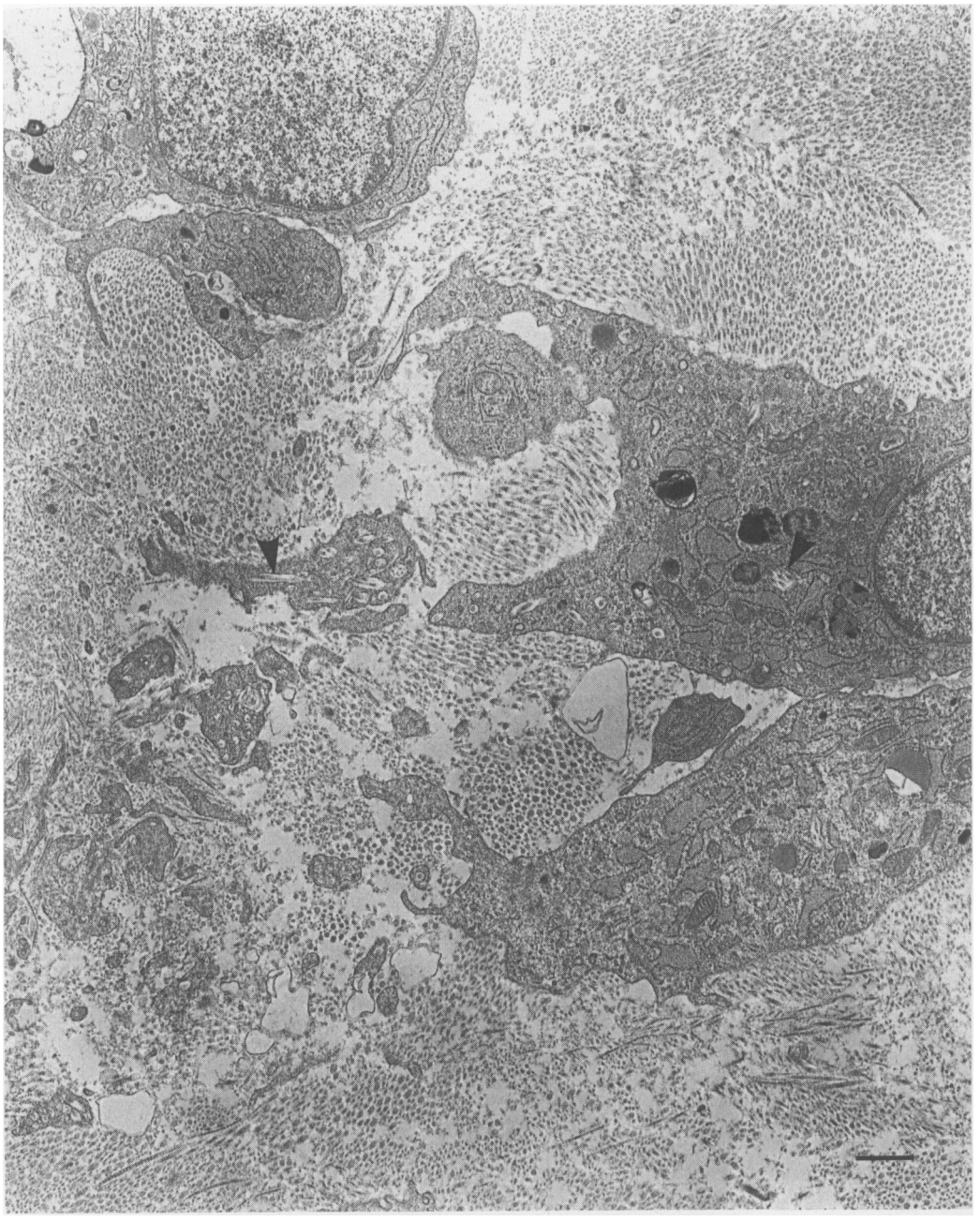

Fig. 8. Day 12: Synovial macrophages with cytoplasmic extensions and vacuoles containing collagen fibrils (arrows). Note the heterogeneous distribution of collagen diameters in the extracellular space. Bar, 1 μm.

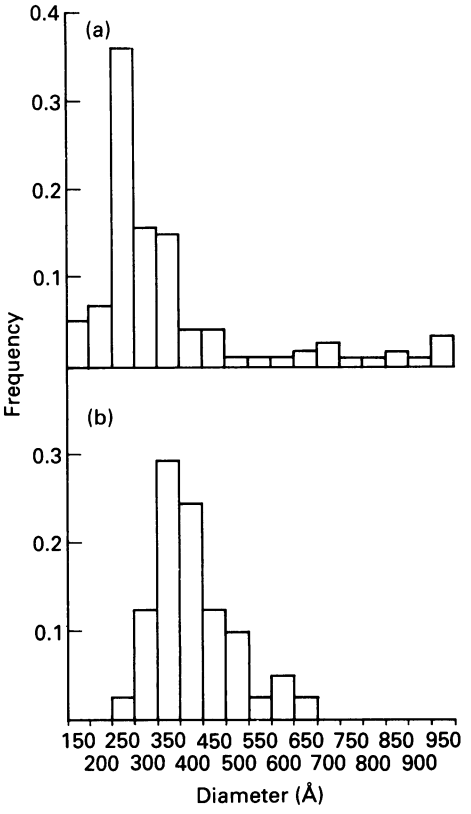

Fig. 9. Day 12: Frequency distribution of collagen fibril diameters obtained from randomly selected areas of synovial tissue in a, experimental (no. of fibrils measured, $n = 800$) and b, control ($n = 700$) joints.

phages or fibroblasts, and that most of the chondrocytes appeared healthy.

Group V (26–27 days; four animals)

At this time, the appearance of the synovium was more variable. In some specimens there was still heavy infiltration of fibrin and lymphocytes, whilst in others this inflammatory reaction was mild. In most specimens cell clustering and enveloping, as observed earlier, could still be seen. In one animal, the deep synovial tissue had heavy inflammatory infiltrates and the cytoplasmic rims of the lipocytes were hypertrophied and showed intense pinocytotic activity.

Where surface cartilage was being degraded and infiltrated by fibroblasts and collagen fibrils, a deeper region of fibrosis was also observed between the subchondral bone and healthy cartilage. For the first time in the study, mineralization of cartilage regions was observed. In these regions the normal, fine striations of collagen fibrils were absent; instead, the matrix appeared coarsely striated as if the gap regions of the fibrils were being obscured with mineral deposits.

Discussion

Antigen-induced arthritis in the rabbit is a chronic monoarticular lesion which can last the lifetime of the animal. There is rapid loss of proteoglycan from the articular cartilage in this lesion (Pettipher *et al.* 1989a). This loss appears to be due to activation of resident cells since in leukopenic animals there is no diminution of cartilage degradation (Pettipher *et al.* 1988). Furthermore, progressive cartilage destruction requires the participation of cell-mediated immune mechanisms (Pettipher & Henderson 1988). Pannus is a feature of this model and is reported to be present within 1–3 weeks (Dumonde & Glynn 1962). Indeed, it is common to see thickening of the synovial lining and continuing overgrowth of this tissue onto cartilage as this experimental lesion develops. In this study we have followed the development of pannus using electron microscopy over the first 4 weeks of the development of arthritis. Tissue was taken from the chondrosynovial junction at the lateral border of the lateral femoral condyle. The synovium overlies both articular hyaline cartilage and condylar bone in this region. We have concentrated our attention on the synovium–cartilage interface.

An ultrastructural study of this kind poses the usual problem of sampling. The experiment was designed to get the maximum information about the time course of the lesion, whilst accommodating biological variability and the practical limits of transmission electron microscopy. Accordingly ani-

mals were killed in five groups corresponding to time spans which were considered to reveal stages in development of the pathology; in particular, changes were sought which were apparently representative of a particular time span and which clearly differed from the previous time group.

Extracellular matrix of articular cartilage may be lost by at least of three proposed mechanisms. Cells in pannus may remove matrix locally at the advancing tissue interface. Lytic enzymes in synovial fluid may digest the cartilage surface matrix at sites distant from synovial tissue or pannus. Matrix may be degraded within the cartilage through the agency of chondrocytes under the influence of soluble factors released from cells in the synovium (reviewed in Henderson & Edwards 1987; Henderson *et al.* 1987). The findings of this study support the involvement of all three mechanisms. Furthermore, it was striking how rapidly events progressed at the chondrosynovial junction of inflamed joints. Pannus was found to encroach progressively over and under the cartilage with an increasingly ill-defined interface between soft tissue and cartilage. This was in part due to the fibrotic reaction occurring in the inflamed synovial lining. This turned the easily recognizable adipose synovial lining into one which at the ultrastructural level was difficult to discriminate from articular cartilage. The cartilage surface also became irregular at sites not directly overlain by pannus. It was at these sites that amorphous aggregates of material of moderate electron density were seen, which material may correspond to previously observed aggregates of immune complexes and cartilage matrix degradation products reported by others (Cooke & Jasin 1972). As an example of the rapidity of changes in articular cartilage it was found that as early as 6 h after joint challenge the cartilage contained clusters of large-diameter collagen fibrils, often found in association with necrotic cells. These fibrils were also found in animals killed at 3–6 days duration of arthritis but were not observed in animals with more prolonged disease. The collagen fibrils in the synovial lining of inflamed joints also had different size distributions from that in normal synovium (Fig. 9). Structurally altered collagen can result from a number of defects in the biosynthetic pathway: (i) a defect in expression of one of the polypeptide chains can interfere with the formation of the triple helix; (ii) a defect in crosslinking enzymes can cause malformation of the fibril; (iii) alterations in the local environment can impair fibrillogenesis. It is not possible here to determine which of these could have been the reason for the large fibrils. There is recent evidence that collagen packing and fibrillogenesis can be influenced by proteoglycans (Katz *et al.* 1986), dermatan sulphate (Scott & Orford 1981) and Type IX collagen itself (Poole *et al.* 1988). While the possibility exists that these large fibrils are associated with chondrocytes with an abnormal production of Type IX collagen, it is unlikely due to the short period within which this occurred after induction.

In confirmation of the reported histology and ultrastructure of the human chondrosynovial junction in RA (Kobayashi & Ziff 1975; Bromley & Woolley 1984) the same area in rabbits with arthritis of 3–6 days duration contained cells with the ultrastructural appearance of macrophages. These cells contained intracellular and intracytoplasmic matter, possibly ingested matrix materials. At later times macrophages could clearly be seen with collagen fibrils present in intracellular vacuoles. By day 12–13 the interface between the cartilage and the synovium had all but disappeared and there was a mixed cell population in the pannus consisting of fibroblasts, plasma cells, lymphocytes, macrophages and polymorphonuclear leucocytes. These cells were closely packed. Close association between macrophages and lymphoid cells is a common feature of rheumatoid synovitis and has been observed in peroxidase-induced arthritis in rabbits (Graham & Shannon 1973). This raises the possibility that the macrophages are acting as antigen-presenting accessory cells (Cohn

1968). However, in this study macrophages were often seen surrounding plasma cells. It is noteworthy that both in the T-cell zone of human tonsil and in T-cell clusters in rheumatoid synovium two quite distinct types of cell interdigitate between the lymphocytes. One type carries the macrophage marker EBM11 but not the HLA-DQ related marker RFD1, and the other type, with rather more extensive dendrites, carries the reverse phenotype (Wilkinson and J.C.W. Edwards, unpublished observations). The former appears to be a macrophage and the latter a true interdigitating dendritic cell. Although both cells may have an accessory antigen-presenting function it is equally possible that the macrophages have a cytotoxic or scavenging role.

Cells of the 'interdigitating dendritic cell' or 'veiled cell' type may be of crucial importance to persistent inflammation in human rheumatoid synovitis. These cells are not unambiguously identifiable in tissue sections by light microscopy but have a typical ultrastructure and have been identified in rheumatoid tissue and fluid by electron microscopy (Harding & Knight 1986). A specific search for cells of this type was made but none were found. This may simply be a sampling problem or an indication of the relatively short time-span of the model, but suggests that such cells are not present in abundance. It remains to be seen if these cells are present in an extended study of the model so that a more meaningful comparison can be made with truly chronic human cases. If they are absent, this could indicate an important difference in the type of immune response between the rabbit and human lesions and support the view that the presence of large numbers of such cells in human synovitis is an indication of underlying immune dysregulation.

The finding of small blood vessels with tall endothelial cells reflects similar findings in rheumatoid synovium (Freemont *et al.* 1983; Iguchi & Ziff 1986). However, in the rabbit lesion, these do not necessarily appear to be associated with lymphocyte aggregates. This favours the idea that the development of tall endothelium occurs in response to signals from macrophages or fibroblasts rather than lymphoid cells already present in the lesion.

In summary, the ultrastructure of AIAR supports previous light microscopic findings. Extremely rapid changes in the collagen fibrils of the extracellular matrices of synovial lining and articular cartilage were noted. In particular, collagen fibrils with unusually large diameters were observed in the cartilage. Overgrowth of the cartilage by the synovial lining was seen within 3–6 days of induction of arthritis and by 12 days it was impossible to recognize the interface between these two tissues. The synovial pannus at this time was fibrotic and contained large numbers of plasma cells, lymphocytes and macrophages. Few polymorphonuclear leucocytes were found in the developing pannus. Macrophages were found with extended processes which enveloped neighbouring cells. Some blood vessels had thickened walls resembling high endothelial venules, though lymphocytes were not observed in their vicinity. This study goes some way towards unravelling mechanisms of cartilage matrix loss. The absence of cells of interdigitating dendritic ultrastructure may indicate an important difference from the human immunopathology.

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