CLEARANCE OF Fe^{59} -LABELLED ERYTHROCYTES FROM NORMAL AND INFLAMED RABBIT KNEE JOINTS

II. AUTORADIOGRAPHIC AND HISTOLOGICAL STUDIES

BY

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These experiments have been designed to test the hypothesis that iron present in deposits in the rheumatoid synovial membrane is bound or sequestered in this site (Muirden and Senator, 1968). The experimental model used was the arthritis induced in the rabbit's knee by injections of carrageenin (Gardner, 1960). Labelled red cells were injected into the inflamed and normal knee and a previous paper reports the clearance of Fe^{59} measured by surface counting and by liquid scintillation of ashed segments of membrane (Muirden, 1969). The distribution of iron within the joint tissues was estimated at various stages after injection by histological and autoradiographic techniques, and these studies form the basis of this report.

Material and Methods

Carrageenin arthritis was induced in the right knee joint of rabbits whilst the left knee received saline injections to act as a control. The method of preparing Fe^{59} -labelled red cells and their introduction into the two joints has already been described (Muirden, 1969). The rabbits used for histological studies were killed 1, 3, 7, 14, and 42 days after injection of the erythrocytes. The knee joints were opened and rinsed out with saline, and synovium was dissected from the lateral and posterior areas of the joint. Samples were also taken from menisci and articular cartilage.

Tissue for routine histology was fixed in neutral, phosphate-buffered 10 per cent. formalin and sections were stained with haematoxylin and eosin and by the Prussian blue method for iron. Tissue for autoradiography was fixed in Carnoy's solution (absolute alcohol, chloroform, and glacial acetic acid) for 30 min. to 3 hrs, then transferred to absolute alcohol, cleared in chloroform, and embedded in paraffin. Glass slides cleaned in chromic acid and rinsed in distilled water were immersed in a solution of gelatin and then dried. Sections were cut at 5 to 6 μ and fixed to the slides. Kodak

photographic emulsion (NTB-2) was heated in a water bath at 42°C. for 1 hr. The slides were immersed for 2 sec. and then drained. When dried the slides were stored at 4°C. in light-proof boxes for the exposure period which was generally 3 or 6 weeks. After the photographic emulsion had been developed and fixed the sections were stained with Unna-Pappenheim solution.

Results

Macroscopic Changes

Blood staining of the synovial membrane was obvious in rabbits killed 1 and 3 days after injection of red cells and at 7 days the membrane had a golden-brown appearance. The synovial cavity of the carrageenin injected joints contained excess fluid and the synovium was thickened. These changes were still present 42 days after injection but the left control joint was then free of pigment and appeared normal. Evidence of blood seepage along the course of the extensor digitorum tendon, which in the rabbit arises from within the joint capsule, was visible on both sides.

Microscopic Changes

The inflammation induced by carrageenin features synovial proliferation and cellular infiltration (Fig. 1, opposite). Red cells are occasionally seen in the inflammatory exudate and patchy haemosiderin deposits have been described (Muirden and Peace, 1969).

1 day after injection of labelled red cells.—Haematoxylin and eosin sections showed red cells mainly within folds of the membrane. Haemosiderin granules were not seen, Prussian blue stains were essentially negative, and autoradiographic sections failed to show significant counts above background on the two sides. It appeared that the macroscopic blood staining was due to surface cells and most had been washed away with the saline

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Fig. 1.—Carrageenin arthritis. The proliferated synovium is infiltrated with inflammatory cells. Giant cells are seen in the deeper stroma. Haematoxylin and eosin. $\times 200$.

Fig. 2.—Control joint, Day 3. Red cells are present in the lumen and are infiltrating the mildly proliferated lining layers (arrow). Haematoxylin and eosin. $\times 800$.

irrigation. The irritative effect of the haemarthrosis was evidenced by increased numbers of polymorphonuclear leucocytes within the synovium.

3 days after injection.—In the left control knee numerous red cells were seen infiltrating the mildly proliferated synovium (Fig. 2). An occasional macrophage just beneath the lining layer contained haemosiderin granules and neutrophils and lymphocytes were also present.

Iron stains showed pale blue staining of some surface cells and dense staining of stromal macrophages. In the right carrageenin-injected joint, iron staining was more diffusely spread through the surface layers of the thickened membrane. The control joint autoradiographs, despite high background counts, showed increased grains in relation to the surface cells and more particularly just beneath the lining layer (Fig. 3, overleaf). In the carrageenin joint, background counts were much lower and more precise localization of Fe^{59} was possible (Fig. 4, overleaf).

Labelling was present in relation to the surface cells, but in a greater concentration three or four cells deep to the lumen. Some of the intense labelling corresponded to haemosiderin in macrophages. The deeper parts of the stroma were largely free of the label.

7 days after injection.—Fewer red cells were seen in both joints although erythrophagocytosis was rarely visible. Haemosiderin containing macrophages were much more numerous. Prussian blue stains showed iron in scattered surface cells as well as in the macrophages in the left control joint (Fig. 5, overleaf). Iron staining was more widely dispersed in the right carrageenin-injected knee and some dense blue staining appeared to be extracellular. Autoradiography showed labelling of surface cells and dense grain patterns in the superficial stroma in the control knee. In addition to this the carrageenin synovium showed heavy labelling of deeper areas of the membrane (Fig. 6, overleaf).

Fig. 3.—Control joint, Day 3; autoradiograph. Increased grains over background are present in relation to lining cells but more especially in the superficial stroma. $\times 900$.

14 days after injection.—The left control synovium now showed only slight lining cell proliferation, the infiltrate of red and white cells had disappeared, and only a few of the stromal macrophages contained iron. In the autoradiographic slides few of the lining cells showed increased counts above background, but very dense labelling was seen over

Fig. 5.—Control joint, Day 7. Surface cells and large macrophages just beneath the lining layer show dense iron deposits. Prussian blue. $\times 800$.

macrophages just deep to the surface (Fig. 7, opposite).

In the right carrageenin-injected joint the striking feature was the presence of large conglomerate masses of iron, much of which appeared to be extracellular (Fig. 8, opposite). Other areas of the

Fig. 4.—Carrageenin arthritis, Day 3; autoradiograph. The label is concentrated into cells just beneath the surface layer. $\times 1,120$

Fig. 6.—Carrageenin arthritis, Day 7; autoradiograph. Dense labelling is seen in focal collections in the deeper stroma. Lining cells are also labelled but to a lesser degree. $\times 800$.

Fig. 7.—Control joint, Day 14; autoradiograph. Background counts are high but dense labelling is seen over macrophages in the superficial stroma. $\times 800$.

Fig. 8.—Carrageenin arthritis, Day 14. Large conglomerate extracellular masses of iron are seen within the proliferated synovium. Prussian blue. $\times 400$.

synovium adjacent of these deposits were quite free of iron. Intense Fe^{59} labelling was seen over stromal macrophages and over the extracellular haemosiderin masses (Fig. 9).

Fig. 9.—Carrageenin arthritis, Day 14; autoradiograph. Concentrations of grains are seen over extra-cellular haemosiderin masses in the synovial stroma. $\times 1,120$.

42 days after injection.—Material was available only for histological studies. The left control synovium was normal apart from a few stromal macrophages containing iron. On the right the membrane was proliferated, and patchy deposits of iron, including extracellular masses, were still to be seen. Less iron was present than at the 14-day stage.

At each stage sections of articular cartilage and meniscus failed to show iron on histological or autoradiographic studies.

Discussion

Muirden (1969) demonstrated that the radioactive clearance after intra-articular injections of Fe^{59} -labelled erythrocytes was slow, and the decay from joints inflamed by previous treatment with carrageenin was even more prolonged. Histological

studies reported here indicate that red cells were prominent within the synovium up to 3 days after injection but were absent at Day 7 when surface counts showed that only one-third of the radioactivity had disappeared from the control joints. Stainable iron was present at Day 3 and was more marked at Day 7 in the synovial lining cells and as haemosiderin in macrophages in the superficial stroma. At Day 14 there was less iron, and 42 days after injection only a few haemosiderin-containing macrophages were noted. Autoradiography showed that peak labelling was maintained through to the 7-day stage when red cells had disappeared from the membrane. It appeared, therefore, that few erythrocytes could have escaped intact from the synovium. The rarity of erythrophagocytosis in experimental haemarthrosis has been commented on by Roy and Ghadially (1966) but there is good electron microscopic evidence that this does occur. An estimation that phagocytosis and digestion of red cells by reticular cells takes only 10 minutes (Bessis and Breton-Gorius, 1962) may explain the difficulty in showing the process histologically.

The autoradiographic studies were of importance in the carrageenin-injected joints where iron deposits can occur as the result of the inflammatory exudate (Muirden and Peace, 1969). The label was found to be spread in a patchy fashion throughout the thickened membrane. Sampling problems associated with the irregular distribution of Fe^{59} may explain a peak level of radioactivity at Day 7 rather than Day 3 as in the control joint. In contrast to the control, a high level of radioactivity was maintained at Day 14 and was associated with extracellular masses of haemosiderin and these were still present 42 days after the injection of erythrocytes. The distribution of iron in relation to the time elapsed is evidence that the deposits gradually accumulate from the joint space and not from re-circulating iron. However, whatever the source of iron and accepting possibly different rates of breakdown of incompatible rabbits' red cells, it is clear that the chronically inflamed tissue masses accumulate and fix a considerable amount of iron. The similar distribution of iron in the carrageenin synovium, in human haemarthrosis, and in rheumatoid arthritis favours the joint cavity as the source of iron sequestered in the synovial membrane.

The catabolism of haemoglobin within macrophages releases iron and this is transformed into ferritin (Richter, 1957). This also occurs in synovial cells *in vitro* (Muirden, Fraser, and Clarris, 1967). The pale blue staining of synovial lining cells 3 days after the injection of blood is explained

by this release of ferritin. The capacity of cells to store ferritin is limited and when the concentration reaches a critical level haemosiderin is formed by a process of denaturation (Shoden and Sturgeon, 1962). In the synovium the diffuse blue staining of lining cells gradually disappeared and haemosiderin-containing macrophages became more prominent just beneath the lining layer. This process, whereby iron originally present in many surface cells is concentrated into a few cells on or just beneath the surface, was shown in experiments in which iron-dextran was injected into joints (Ball, Chapman, and Muirden, 1964). From a study of electron micrographs we concluded that this concentration took place primarily by the ingestion of degenerate iron-containing cells by other macrophages. The further step to the breakdown of iron-laden clumps of macrophages to form the extracellular deposits of haemosiderin is more conjectural.

Large extracellular masses of iron are also seen in rheumatoid arthritis (personal observations) but have not been described in the pathology of haemarthrosis or haemophiliac arthropathy, or in the synovial changes in haemochromatosis (*e.g.* Collins, 1949; Schumacher, 1964). Iron excess does seem able to cause tissue damage as has been shown in haemochromatosis (Bothwell and Finch, 1962). It is possible, however, that the chronic inflammatory exudate in rheumatoid and carrageenin arthritis provides an additional factor responsible for cell destruction which is not present in these other conditions. In both rheumatoid and carrageenin arthritis synovial macrophages contain enlarged lysosomes (Muirden and Peace, 1969). The combination of excess phagocytosis in cells showing lysosomal abnormalities must be conducive to lysosomal enzyme release and to cell destruction.

The release of iron from insoluble haemosiderin complexes in these extracellular masses is likely to be slow. Deposits were still evident 42 days after the injection of blood at a time when iron in the control joint was confined to a few stromal macrophages. Additional evidence that iron in very large aggregates of haemosiderin is relatively unavailable

is provided by Beutler (1958).

Another possible reason for the slow clearance of iron concerns the lymphatics of the synovial membrane. Small molecules leave the synovial cavity rapidly *via* the blood, but it has long been held that proteins and colloidal particles are removed mainly by lymphatics (Bauer, Short, and Bennett, 1933; Adkins and Davies, 1940). The latter have shown that particles of greater diameter than 0.1μ have no route out of the subsynovial tissues. The effect of inflammation on this process is likely to depend on the acuteness or chronicity of the reaction. Unlike capillaries fine lymph vessels terminate at some distance from the articular margin and are absent from synovial villi (Davies, 1946). The swollen hypertrophied synovium in carrageenin arthritis may provide a mechanical barrier to the clearance of larger molecules and particles which would not apply to small molecules which leave by the capillaries.

Summary

Autoradiographic and histological studies of the synovial membrane have been made after the injection of Fe^{59} -labelled erythrocytes into normal rabbit joints and into joints inflamed by previous treatment with carrageenin. Differences were found in the distribution and disappearance rate of iron. In the control joints peak levels of radioactivity were found at Day 3 and Day 7 after the injection, at a time when red cells were disappearing and haemosiderin was appearing in synovial macrophages. In the carrageenin-injected joints high grain counts were maintained until Day 14, and even at Day 42 sizeable deposits of iron were still visible. The presence of large extracellular deposits of haemosiderin in the carrageenin joints similar to those noted in rheumatoid arthritis is one reason suggested for the slower clearance from the inflamed joint.

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**L'élimination des érythrocytes marqués par le Fe^{59}
de l'articulation normale ou enflammée du lapin
II. Les études autoradiographiques et histologiques**

RÉSUMÉ

Les études autoradiographiques et histologiques de la membrane synoviale ont été faites après l'injection d'érythrocytes contenant du Fe^{59} dans les articulations normales du lapin et dans les articulations enflammées par traitement antérieur avec de la carragheen. Des différences ont été remarquées dans le taux de la distribution et la disparition du fer. Dans les articulations témoins les plus hauts niveaux de radioactivité ont été trouvés le troisième et le septième jour après l'injection, au moment où les globules rouges disparaissaient et l'hémossidéine apparaissait dans les macrophages synoviaux. Dans les articulations ayant reçu la carragheen, l'énumération des grains était élevée jusqu'au quatorzième jour, et même au quarante-deuxième jour, des dépôts de fer d'une certaine grosseur étaient encore visibles. La présence de larges dépôts extra-cellulaires d'hémossidéine dans les articulations ayant reçu la carragheen semblables à ceux trouvés dans la polyarthrite rhumatoïde est une raison qui a été suggérée pour expliquer l'élimination plus lente de l'articulation enflammée.

**Eliminación de eritrocitos identificados con Fe^{59} en
articulaciones de rodilla de conejo, normales e inflamadas
II. Estudios autorradiográficos e histológicos**

SUMARIO

Se han realizado estudios autorradiográficos e histológicos de la membrana sinovial, después de haber inyectado eritrocitos identificados con Fe^{59} en articulaciones normales de conejo y en articulaciones inflamadas por tratamiento previo con carragaen (musgo de Irlanda). Se hallaron diferencias en la distribución y promedio de eliminación del hierro. En las articulaciones testigo, los niveles más altos de radioactividad se notaron al tercero y séptimo días después de la inyección, al tiempo que desaparecían los glóbulos rojos y aparecía hemosiderina en los macrófagos sinoviales. En las articulaciones inyectadas con carragaen se mantuvo un alto contenido de gránulos hasta el decimocuarto día, y aún 42 días después, todavía eran visibles depósitos de hierro de tamaño considerable. La presencia de grandes depósitos extracelulares de hemosiderina en las articulaciones tratadas con carragaen, similares a aquellos descubiertos en la poliartritis reumatoide, es una de las razones que se sugieren para explicar la lenta eliminación en la articulación inflamada.