INTRACELLULAR COLLAGEN IN EXPERIMENTAL ARTHRITIS IN RATS

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In recent years the development of improved autoradiographic techniques and in particular electron micro-autoradiography (Caro and von Tubergen 1962) has led to a better understanding of collagen fibre production by fibroblasts (Revel and Hay 1963, Ross and Benditt 1965). It is now generally accepted that collagen fibres are formed outside the cell by an extracellular aggregation of soluble collagen precursors (Gross 1956, Revel and Hay 1963, Goldberg and Green 1964, Ross and Benditt 1965). It appears that the soluble precursor (tropocollagen) is released from the cell probably through the vesicles along the cell border. Once extracellular, the tropocollagen aggregates into fibrils which gradually increase in diameter until the definitive collagen fibres are formed.

Experiments on rats with an induced adjuvant polyarthritis have shown certain unusual features in the cells of the proliferating granulation tissue excised from about the affected joints. Cells, which had the electron microscopic characteristics of fibroblasts, appeared to contain numerous inclusions, and prominent among these inclusions were bundles of collagen fibres. To determine the origin of these fibres the synthesis of collagen by these cells was studied by autoradiographic techniques, using tritiated proline as a label.

MATERIALS AND METHODS

The animals were adult “hooded” rats weighing between 150 and 250 grammes. The adjuvant was a modified Freund’s adjuvant (Gardner 1968) and consisted of mixed strains of heat-killed and freeze-dried Mycobacteria tuberculosis, 50 milligrams ground and mixed in 10 millilitres of paraffin. The polyarthritis was produced by an intradermal injection of 0.05 millilitre of the mixture into the skin of the right fore paw of the rat. The disease developed after a latent period of fourteen to twenty-one days and mainly affected the more peripheral joints of all four limbs.

Electron microscopy—Small pieces of para-articular tissue were taken at weekly intervals from paws affected by adjuvant arthritis. The tissue was fixed in cold 3 per cent glutaraldehyde in a 0-1 molar cacodylate buffer at pH 7-4, post-fixed in osmium tetroxide, dehydrated in graded ethanol series and stained with phosphotungstic acid. The tissue was then embedded in Araldite; silver sections were taken and mounted on a formvar-covered grid. For greater cellular detail some of the sections were stained on the grid with uranyl acetate (saturated solution in 50 per cent ethanol, fifteen minutes at room temperature), and lead citrate (Reynolds 1963); the solutions were filtered by the method of Rowden (1969).

Autoradiography—Four weeks after the injection of adjuvant six rats were injected with 0.5 millicurie of tritium-labelled proline, into the peritoneal cavity. These rats were killed at ten, fifteen and thirty minutes, one, two and six hours. Para-articular tissue taken for autoradiography was fixed in 10 per cent formalin, decalcified in ethylene-diamine-tetra-acetate and embedded in wax; sections were cut to six microns. The autoradiographs were prepared by a stripping film technique using Kodak Autoradiographic Stripping film AR 10. The autoradiographs were developed at three weeks and the sections stained with methyl green and pyronin.

RESULTS

Electron microscopy—The majority of cells were oval or spindle-shaped, although a wide variety of shapes was seen. Most of these cells were recognised as fibroblasts from the various intracellular features described as characterising this type of cell (Ross 1968).
Fig. 1
A fibroblast six weeks after the injection of adjuvant. (Phosphotungstic acid, × 6,500.) Note the endoplasmic reticulum (er), nucleolus (n) and intracellular fibrils (f), some of which are cut in transverse section (t, bottom right and inset). (Inset, × 19,500.)

Fig. 2
A fibroblast four weeks after the injection of adjuvant. (Phosphotungstic acid, uranyl acetate, lead nitrate, × 6,500.) Note the prominent nucleus with double-layered membrane, endoplasmic reticulum (er), mitochondria (m) and fibrils (f).
The nucleus was large and prominent (Fig. 1), a characteristic of cells active in protein synthesis (Perry and Errera 1960). A prominent endoplasmic reticulum was seen in most sections (Figs. 2 to 4), particularly in those stained with uranyl, acetate and lead citrate. The reticulum took the form of long narrow cisternae which contained cytoplasm more homogeneous and more dense than the surrounding cytoplasm (Fig. 4). In some cells peripheral vesicles were well marked (Fig. 5) and in others cyclofilaments were prominent (Figs. 3 and 5). The majority of cells contained numerous and prominent mitochondria (Figs. 3 and 4). The most
striking feature about these fibroblasts was the numerous intracellular inclusions. In many of the cells there were large numbers of collagen-like fibres (Figs. 1, 2, 3 and 6). Most of the fibres lay parallel to the long axis of the cell, but some were at right angles to it (Fig. 1).

**Fig. 5**
A fibroblast three weeks after the injection of adjuvant. (Uranyl, lead citrate, ×20,500.) Note the peripheral vesicles (v) and cyclofilaments (c).

**Fig. 6**
A fibroblast three weeks after the injection of adjuvant. (Uranyl acetate, lead citrate, ×26,900.) Note the collagen (f) within the vacuoles and the electron dense material.

Most appeared to lie within vacuoles surrounded by a membrane (Figs. 1 and 6). Numerous other and at present unidentified electron-dense bodies were present within the cells (Figs. 2 and 6).
Fig. 7
Adjuvant arthritis at three weeks. (Methyl green, pyronin, ×1,200.) Autoradiography of granulation tissue (0.5 millicurie tritiated proline) fifteen minutes after intraperitoneal injection. Note that the greatest number of silver grains is intracellular.

Fig. 8
Adjuvant arthritis at three weeks. (Methyl green, pyronin, ×1,200.) Two hours after intraperitoneal injection. Note that the greatest number of silver grains is now extracellular.
Autoradiography—At ten minutes there was a patchy distribution of reduced silver grains, mainly over the more vascular areas of granulation tissue. The grains were almost entirely over the cells and only an occasional grain was seen overlying the intercellular matrix (Fig. 7).

![Graph showing log of ratio of intracellular to extracellular grain counts over time.](image)

**Fig. 9**
The ratio of intracellular to extracellular grain counts in the granulation tissue of rats with adjuvant arthritis, following the intraperitoneal injection of 0.5 millicurie of tritium-labelled proline. Until one hour after the injection the greater number of grains is over the cells; after one hour the greater number of grains is over the matrix.

![Diagram showing the area A.](image)

**Fig. 10**
The area (A) is in the granulation tissue adjacent to the paracortical new bone and is the area where the grains were counted.

After fifteen minutes the ratio of grains overlying cells, compared with grains over matrix, fell, and at two hours the maximum number of grains was seen over the matrix (Fig. 8). The ratio of the total number of grains overlying cells to the total overlying the matrix at
different times after the injection of proline 3-H is shown in Figure 9. To enable areas of comparable activity to be counted, the counts were taken in the areas of maximum activity in the granulation tissue, adjacent to the paracortical new bone which formed in this disease (Fig. 10). At two hours after the injection of labelled proline the majority of the proline was extracellular (Fig. 9).

DISCUSSION

The demonstration of collagen within cells may be explained in three ways. 1) The presence of collagen within the cell is an artefact, tangential sections giving the appearance that the fibres are intracellular. 2) The collagen is produced by intracellular synthesis. 3) The collagen has been ingested by the cells.

Although the possibility of an artefact cannot be completely excluded, the presence of collagen in large numbers of cells, and in some sections the presence of fibres in transverse section (Fig. 1), make it unlikely that in all cases the intracellular collagen was an artefact. In many sections the fibres appeared to extend deeply into the cytoplasm, and often the sections included a complete nucleus and Golgi apparatus, suggesting that they were cut at approximately the mid-point of the cell.

One of the difficulties presented in this study has been the classification of the cells in the granulation tissue, and in particular those cells which contain collagen fibres. Fibroblasts and macrophages cannot be distinguished by light microscopy alone, and the distinction can be made with any degree of certainty only by the electron microscopic examination of the intracellular organelles. Ross and Benditt (1961) suggested that the most striking feature of the fibroblasts compared with macrophages was the presence of an extensive dilated endoplasmic reticulum. In addition, fibroblasts contained enlarged mitochondria with irregular small cristae, intracytoplasmic filaments, occasional irregular amorphous dense bodies, and rarely myelin figures. In contrast the macrophages contained numerous vacuoles, full of ingested matter, myelin figures, irregular dense amorphous masses and characteristically the cisternae of the endoplasmic reticulum of the macrophages were short and narrow. However, even by electron microscopy the distinction between these two cell types is not entirely satisfactory, and Ross and Benditt have suggested that there may be a transition between the two.

Light autoradiography (Figs. 7 and 8) showed that the majority of cells in the granulation tissue were rapidly taking up proline. Assuming that proline is utilised predominantly for collagen synthesis, the majority of the cells in the granulation tissue appeared to be fibroblasts.

The extensive endoplasmic reticulum of most of the cells seen by the electron microscope (Figs. 1 to 6) confirms the classification of these cells as fibroblasts.

The possibility that collagen can be formed within the cell has been considered for many years. Flemming (1897) and Laguesse (1921) supported this concept, and fibrils have been demonstrated within cells (Wasserman 1954; Yardley, Heaton, Gaines and Shulman 1960; Avery and Han 1961), yet these fibrils have not been shown to be collagen precursors. Welsh and Meyer (1967) demonstrated collagen within the fibroblasts of a number of human tumours and suggested that the collagen was synthesised within the cells. In this study the light autoradiographic studies were of little value in confirming the hypothesis of intracellular collagen synthesis, because the labelling could not be identified with specific collagen fibres.

The present studies, however, do show that if phagocytosis of collagen does occur, it is by cells that are at the same time utilising proline, namely fibroblasts. Phagocytosis of collagen has been reported by many authors. In most cases the collagen has been seen within macrophages, and the collagen has lost the periodic banding and to some extent the fibre definition (Vassos 1940). Intracellular banded collagen has been demonstrated (Luse and Hutton 1964, Parakkal 1969, Dingle 1969), and in the experiments of Luse and Hutton the cells were thought to be fibroblasts. Dingle, using organ culture and feeding the fibrous
tissue high concentrations of sucrose, demonstrated phagocytosis of sucrose and collagen by the fibroblasts. Rojkind and Pérez-Tamayo (1962) demonstrated collagen resorption in implanted autologous rat tendon. The tendon was invaded by fibroblasts, and it appeared that the collagen resorption as well as collagen formation was controlled by the fibroblasts. When autologous tendon was implanted in other situations—in the dog (Flynn and Graham 1962) and in chickens (Lindsay and McDougall 1961)—the collagen was reabsorbed by "undifferentiated cells", and synchronously new collagen was formed by invading fibroblasts.

· Which of the two explanations of intracellular collagen is the correct one may be resolved by electron microscopic radiography, but the presence of other intracellular material (Figs. 2 and 6) suggests that the collagen is present by a process of phagocytosis rather than synthesis. If this is so, then under certain circumstances a fibroblast can perform the dual role of synthesis and phagocytosis. This is consistent with the hypothesis initially proposed by Maximow (1927) and later by Ross and Benditt in 1961 (Ross 1968) that fibroblasts and monocytes are variants of the same cell type with a free transition between the two.

SUMMARY

1. Experimental arthritis was induced in rats by the intradermal injection of modified Freund's adjuvant.
2. The granulation tissue occurring in and around the joints was examined with the electron microscope.
3. Intracellular collagen was demonstrated in many of the cells.
4. Collagen formation by these cells was studied by autoradiographic techniques using tritiated proline as a label.
5. The proline turnover was rapid, as most of the labelled proline had become extracellular one hour after its injection.
6. It was concluded that the collagen was present within the cells as a result of phagocytosis despite the fact that the cells had the electron microscopic features of fibroblasts.

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