WOUND HEALING AND COLLAGEN FORMATION

VI. The Origin of the Wound Fibroblast Studied in Parabiosis

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ABSTRACT

Healing skin wounds were studied in a series of parabiotic rats. The femurs of one parabiont of each pair were shielded whilst both animals were given 800 r from a Co^{60} source. The animals were wounded 3 days after irradiation. Each animal with partially shielded marrow was then given tritiated thymidine intraperitoneally daily while the cross-circulation was arrested by clamping. After the thymidine-^3H had cleared the blood, the clamp was released. Animals were sacrificed, and wounds were prepared for radioautography 1, 2, and 6 days after wounding. In the wounds of the shielded animals thymidine-^3H was observed in epidermis, endothelium, leukocytes, fibroblasts, and mast cells. Only neutrophilic leukocytes, monocytes, and lymphocytes were labeled, as determined by light and electron microscope radioautography, in the wounds of each nonshielded parabiont. None of the many fibroblasts present were found to contain label in the wounds of the nonshielded parabionts through the 6 day period. These observations provide further evidence that wound fibroblasts do not arise from hematogenous precursors and, therefore, must arise from adjacent connective tissue cells.

INTRODUCTION

The origin of fibroblasts in healing wounds has been debated for over 100 years, dating from the original observations of Cohnheim (6). He was able to describe migrating leukocytes as they passed through the walls of blood vessels in inflammatory exudates, and suggested that these leukocytes might become transformed to fibroblasts during the process of repair. Many investigations have been pursued since that time, several of which have tended to support Cohnheim's original suggestion (1, 2, 4, 5, 9, 18, 19, 21–23, 31). In contrast, numerous other studies have suggested the opposite, that is, that blood cells do not have the capacity to transform into connective tissue fiber-forming cells (3, 7, 8, 11–17, 20, 24–26).

Limitations in methodology available at the time have largely precluded the possibility of definitively determining whether or not a fibroblast can be derived from a circulating white blood cell.

The recent studies of Volkman and Gowans (29, 30), using parabiotic animals to demonstrate the hematogenous source of mononuclear cells in inflammatory exudates, have presented the possibility of reexamining the question of the source of the fibroblast in healing wounds. The present report involves a similar approach, in which a series of parabiotic inbred rats have been used to determine whether or not fibroblasts can be derived from circulating leukocytes, as determined

by both light and electron microscope radioautography.

MATERIALS AND METHODS

Lewis inbred rats (approximately 60 g each) were joined in pairs by the method of Tyler and Everett (28). This method provides a junction of the body walls of the two animals via a flap of skin in which a cross-circulation is established. The cross-circulation in the flap can be arrested with the use of a rubber clamp for up to 20 min with no side effects. 2 wk elapsed after surgery, so as to permit adequate healing and cross-circulation to be established. Both animals of each pair were then given 800 r of irradiation from a cobalt-60 machine. This dose has previously been shown to be sufficient to destroy essentially all of the hemopoietic tissues in animals which are not protected. The femurs of one of the animals (A) were shielded during irradiation so that their marrows could serve as the source of the blood cells for both of the animals. That this takes place has been established in previous studies of Tyler and Everett (28). 3 days after irradiation, both animals of each pair were wounded by making a series of linear incisions in the dorsal skin. On the day of wounding and at subsequent daily intervals, the cross-circulation was arrested while one member of each pair (A member) was given an intraperitoneal (i.p.) injection of tritiated thymidine. At the same time, the other member (B animal) was given a large dose of cold or unlabeled thymidine (5 mg i.p.). 20 min later, the clamp was removed and each B animal was given a second i.p. injection of 5 mg of unlabeled thymidine. Animals were sacrificed at 1, 2, and 6 days postwounding. A total of eight pairs of parabionts were used in these experiments; four of them were examined by electron microscope radioautography.

At the time of sacrifice, the wounds were removed and prepared for electron microscopy by fixation in buffered 2% osmium tetroxide for 1 hr and postfixed in 10% neutral buffered formalin for 1 hr. They were dehydrated through a series of graded alcohols and embedded in epoxy resin. The major organs and tissues of the animals were removed at the time of sacrifice and fixed in formalin for examination by light microscopy.

1 μ sections of wounds from each animal were prepared and coated with Eastman NTB-2 emulsion for light microscope radioautography. Thin sections were used for electron microscope radioautography. The radioautographs were prepared by placing the thin sections on a glass slide containing a layer of

FIGURE 1 Each of these micrographs is taken from 6 day old wounds of one of the members of a parabiotic pair.

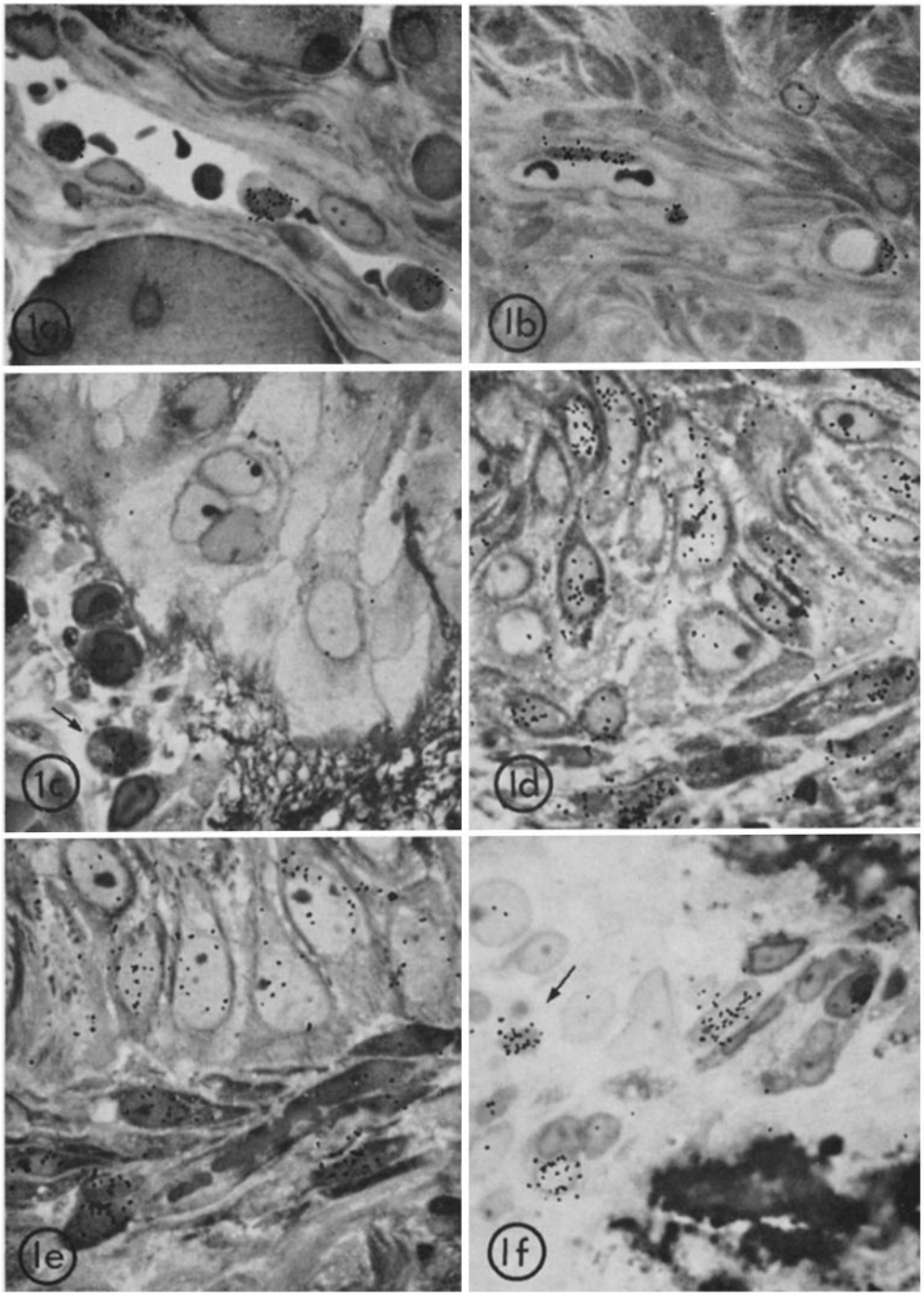
FIGURE 1 *a* Radioautograph of tissue immediately adjacent to the wound of an unprotected (B) animal. Three labeled mononuclear cells can be seen within the lumen of a blood vessel. Such observations were frequent and demonstrate the means of passage of labeled cells from the protected to the unprotected parabiont. × 880.

FIGURE 1 *b* Part of the dermis adjacent to the wound of one of the protected (A) animals. Several labeled endothelial cells as well as a labeled cell in the connective tissue can be seen. This demonstrates the uptake of label by endothelial cells in the protected parabiont in contrast with those of its unprotected mate as seen in Fig. 1 *a*. × 880.

FIGURE 1 *c* This radioautograph should be compared with Figs. 1 *d* and *e*. Part of the regenerated epidermis covering the wound of an unprotected (B) animal can be seen. No label is present in these epidermal cells in contrast with those in the protected (A) animal as seen in Figs. 1 *d* and *e*. A labeled macrophage (arrow) is present in the connective tissue immediately beneath the epidermis. This radioautograph serves as a control to demonstrate that little to no label had crossed over from the protected to the unprotected animal. × 1,160.

FIGURES 1 *d* and *e* Part of the regenerated epidermis from the wound of a protected (A) animal. Both basal cells and spinous cells can be seen to be labeled in these micrographs. Several connective tissue cells, some of which are probably fibroblasts, are also labeled in the subjacent dermis. Fig. 1 *d*, × 1,160; Fig. 1 *e*, × 1,160.

FIGURE 1 *f* This radioautograph shows part of the wound of one of the unprotected (B) animals. Several labeled cells can be seen. One of them can be identified as a macrophage on the basis of a dense body within its cytoplasm (arrow). The others are difficult to identify on the basis of light microscopy. × 1,160.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parlodion covered by a thin layer of carbon. After the sections were placed on the slide, they were dipped, with a dipping machine at a constant rate of speed, in Ilford-L4 nuclear track emulsion and exposed for 6 or 8 wk. The light microscope radioautographs were developed in D-19 and stained with azure-2 methylene blue. The electron microscope radioautographs were developed in D-19b and post-stained with lead tartrate and uranyl acetate. All tissues were examined in an AEI-EM6B electron microscope.

RESULTS

Light Microscope Radioautographs

After 1 and 2 days, wounds from both animals contained large numbers of white blood cells, including neutrophilic leukocytes and monocytes. A majority of the inflammatory cells in the A animals contained grains over their nuclei, and many of these cells in the B animals were similarly labeled.

The significant observations relate to the 6 day time interval after wounding. At this time, the wounds consisted of essentially normal-appearing granulation tissue. They contained large numbers of long, spindle-shaped fibroblasts with prominent nuclei and large nucleoli, together with numerous capillaries. Inflammatory cells including a few neutrophils, lymphocytes, and numerous mononuclear cells were also present. By this time, the epidermis had regenerated and covered the surface of the wound.

The Protected Animal (A)

Cells with labeled nuclei could be seen essentially throughout the wounds of the protected animals which received tritiated thymidine (Figs. 1 *b*, *d*, and *e*). The regenerated epidermal cells contained large amounts of label in the basal cells as well as in the cells of the spinous and granular cell layers (Figs. 1 *d* and *e*). In the underlying connective tissue, cells clearly identifiable as fibroblasts, monocytes, lymphocytes, polymorphonuclear leukocytes, mast cells, and endothelial cells of blood vessels were observed, all of which cells contained label within their nuclei (Figs. 1 *b*, *d*, and *e*).

The Unprotected Animal (B)

Radioautographs of the wounds from the unprotected (B) animals served as controls in that no label was present in the regenerating epidermal cells (compare Figs. 1 *c* and 1 *d*), or endothelial cells (compare Figs. 1 *a* and 1 *b*).

The wounds in the unprotected animals after 6 days were identical in morphological appearance with those in the protected animals. The only cells which were found to be labeled and which were clearly identifiable were mononuclear cells, specifically monocytes or monocytoid cells and lymphocytes. Fig. 1 *a* demonstrates three labeled monocytes within the lumen of a vessel in the dermis adjacent to the wound of an unprotected (B) animal. This clearly shows that labeled cells from the A animal were capable of crossing over to the unlabeled parabiont, and of gaining access to the wounds. The endothelial cells of this vessel are unlabeled, in contrast with those of the protected (A) animal as seen in Fig. 1 *b*. A few cells were observed which could not be easily identified, on the basis of their morphology in the light microscope, as being either fibroblasts or mononuclear cells (Fig. 1 *f*). To further identify these cells, electron microscope radioautographs were prepared.

Electron Microscope Radioautography

Examination of the 6 day old wounds from the protected (A) animal demonstrated the presence of thymidine-^{3}H in all of the cells earlier identified by light microscopy. Fig. 2 contains a labeled cell, identifiable as a fibroblast, from one of the protected (A) animals. The labeled monocyte seen in the wound of an A animal in Fig. 3 could easily have been interpreted to be a fibroblast by light microscopy. The ultrastructure of this cell clearly permits it to be distinguished from the fibroblast seen in Fig. 2.

A careful and thorough examination of all of the blocks of tissue from 6 day wounds of the unprotected (B) animals demonstrated that the nuclei of only three cell types were labeled. These were lymphocytes, monocytes (Fig. 5), and polymorphonuclear neutrophilic leukocytes (Fig. 4). No isotope was found in epidermal cells, endothelial cells, mast cells, or fibroblasts in the wounds of the unprotected (B) animals.

DISCUSSION

A number of different approaches have been used in attempting to determine whether or not connective tissue-forming cells can arise from hematogenous precursors. These approaches included a

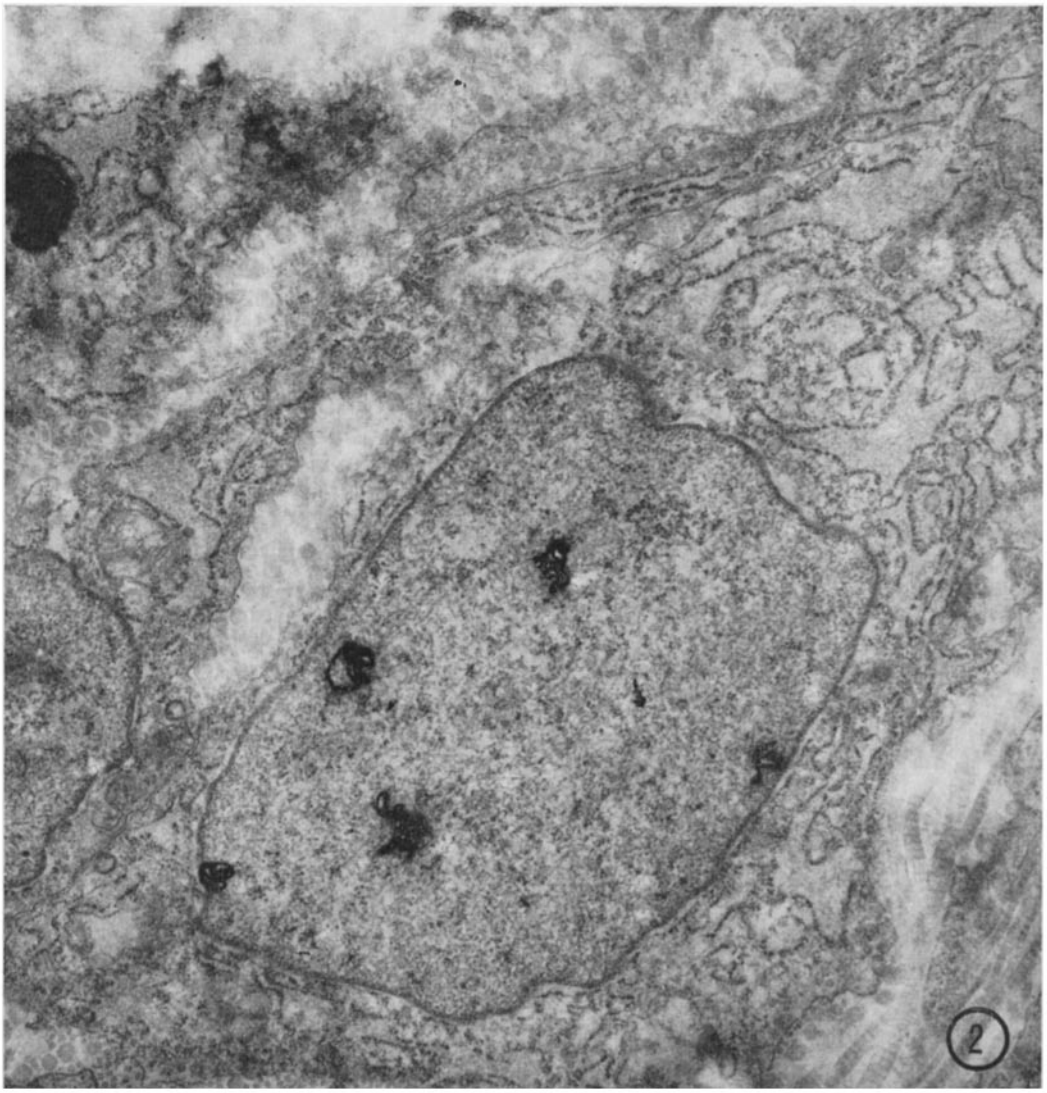

FIGURE 2 This electron microscope radioautograph shows part of a 6 day wound of one of the protected (A) animals. At this level of resolution, this labeled cell can be identified as a fibroblast. Many such cells were present in the 6 day wounds of the protected animals. The abundance and character of the rough endoplasmic reticulum in this cell is one of the features which facilitates its recognition. × 15,000.

study of the fate of explants of buffy coat cells that were grown in tissue cultures, including the studies of Allgöwer and colleagues (1, 2), Rangan (24), Carrel and Ebeling (5), Fischer (9), and Maximow (18, 19). Bloom (4), Hall and Furth (16), and Medawar (20) studied the ability of thoracic duct lymphocytes to make a similar transformation in tissue culture. The ability of cells from peritoneal exudates to become fibroblasts in culture was examined by Moen (21). Similarly, the ability of marrow cells to transform in culture was examined by Farns and Barker (8). Millipore filter diffusion chambers have been used in several studies in which different kinds of cells were grown. Peritoneal cells grown in these chambers were examined by Shelton and Rice (27). Similarly marrow cells were examined by Berman and Kaplan (3) and buffy coat cells were

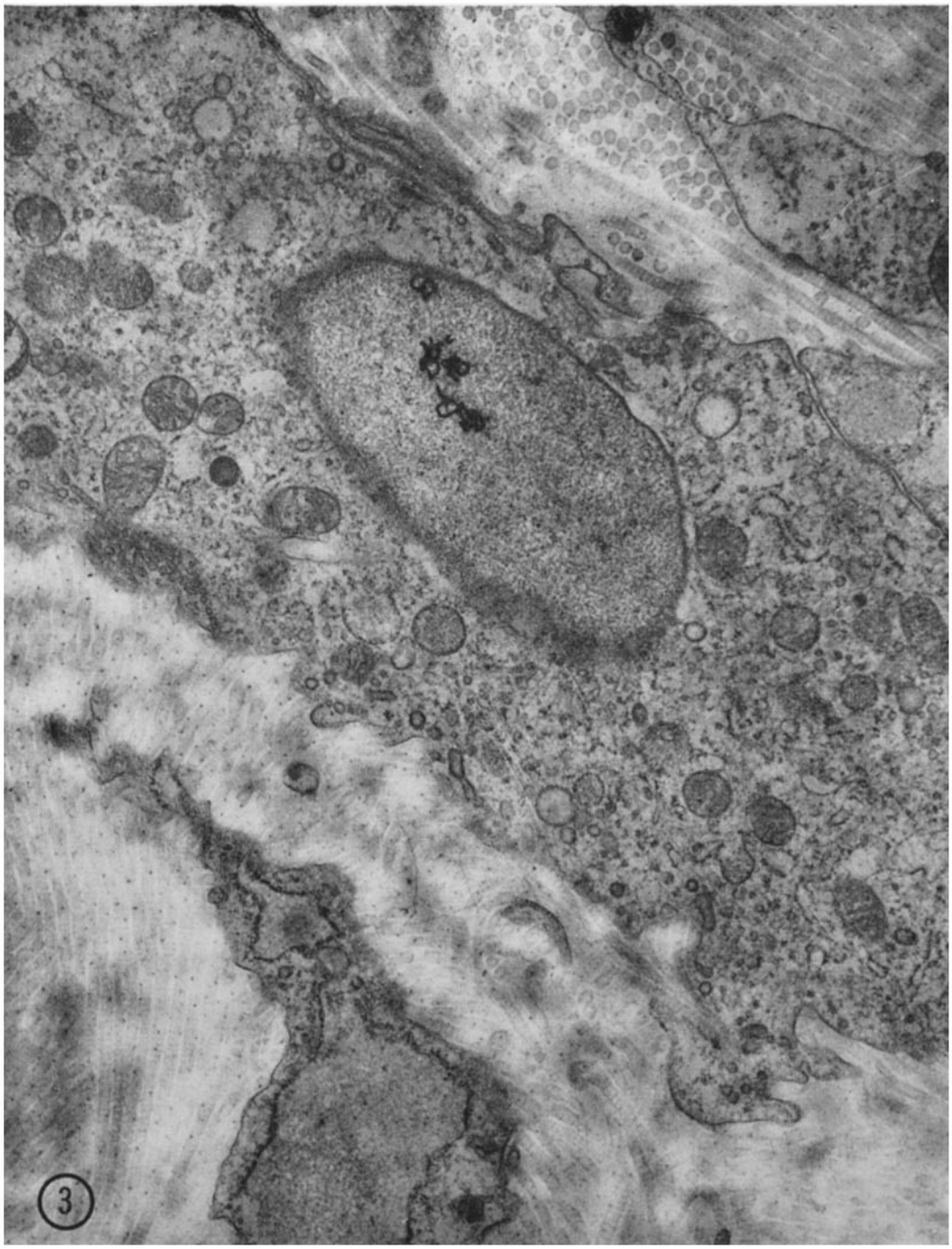

FIGURE 3 This electron microscope radioautograph shows part of a 6 day wound of a protected (A) animal. This labeled cell is a characteristic example of a monocyte. It can be recognized by the relative paucity of rough endoplasmic reticulum and the numerous round, smooth-membrane–bounded structures and vesicles in its cytoplasm. This cell stands in sharp contrast with the fibroblast in Fig. 2. Its elongated appearance, however, would permit it to be confused with a fibroblast in the light microscope. Thus, the use of electron microscope radioautography is an important adjunct in the identification of cell type. × 14,000.

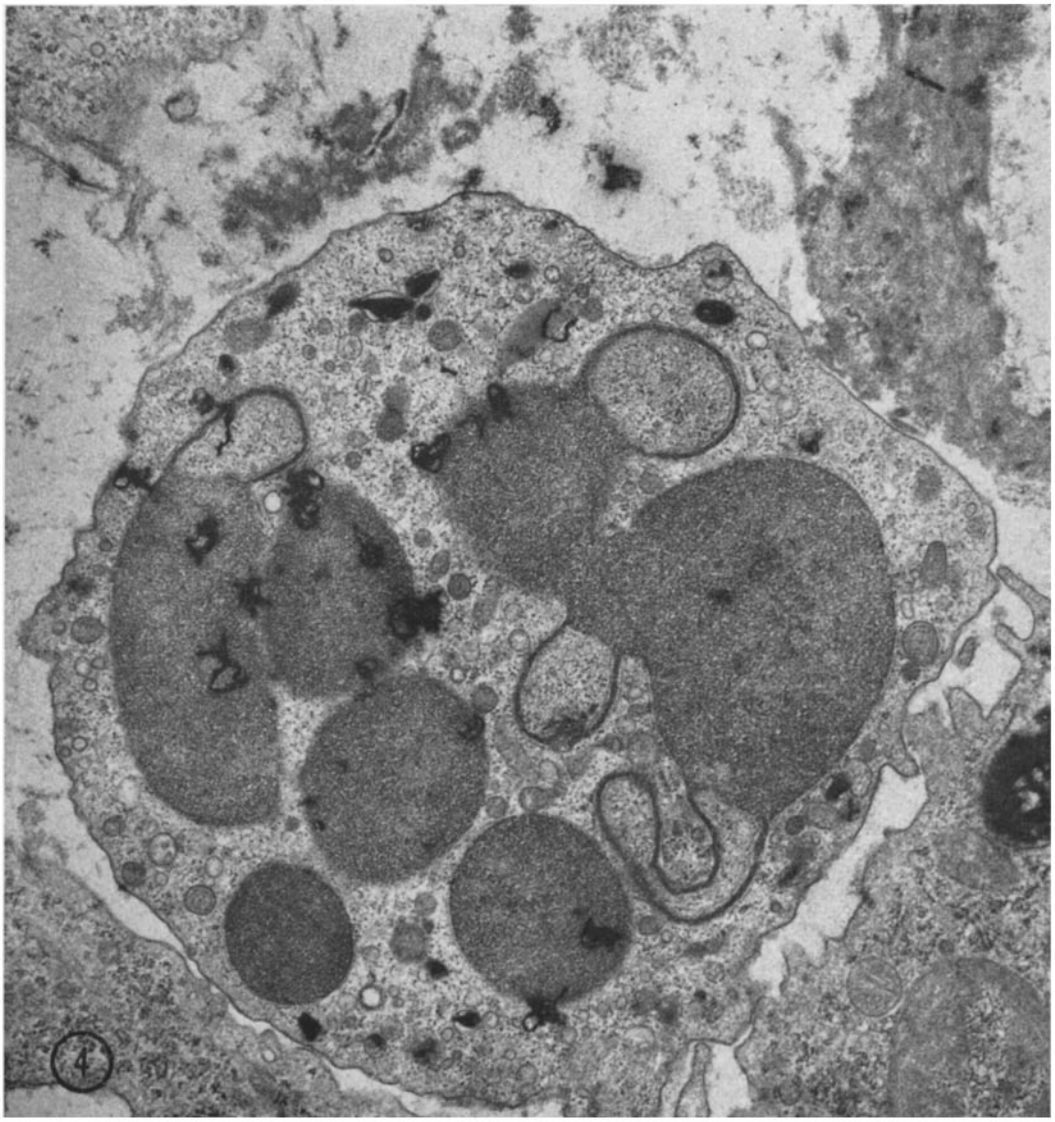

FIGURE 4 This radioautograph was taken from part of a 6 day wound of an unprotected (B) animal. This represents one of the three cell types which contained label in the wounds of the unprotected animals. This labeled cell is a polymorphonuclear neutrophilic leukocyte. × 15,000.

studied by Allgöwer and colleagues (2), Petrakis (22, 23), Rangan (24), and Ross and Lillywhite (26).

A major objection has been raised to those studies purporting to demonstrate a transformation of buffy coat cells to fibroblasts. This resulted from the demonstration that the blood from which the buffy coat cells were obtained was undoubtedly contaminated by a small number of connective tissue cells sufficient to populate the chamber (22–24, 26) or cultures (1, 2) with fibroblasts and collagen. Thus, the question remained unanswered owing to the lack of adequate controls.

Healing wounds also have served as a model for studying the ability of blood cells to become fibroblasts; the studies have involved the utilization of various means of irradiating wounds together with utilization of tritiated thymidine to label

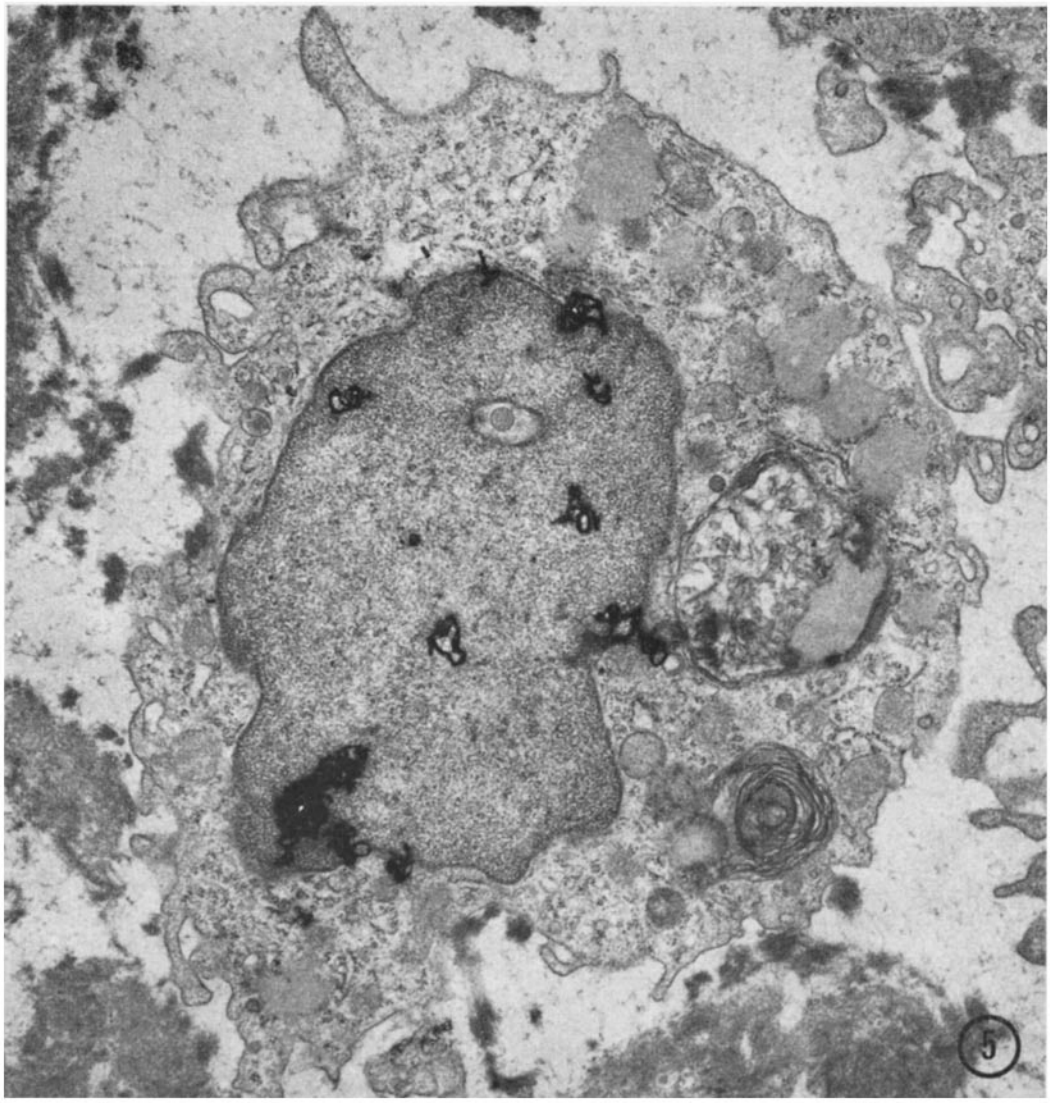

FIGURE 5 The predominant cells found to contain thymidine-^{3}H in the wounds of the unprotected animals after 6 days were monocytes. A representative example of one of these cells is seen in this electron microscope radioautograph. × 15,000.

those cells which come into wounds after they had been irradiated (12–14). In addition, Glücksmann (11) used colchicine to arrest mitosis in wounded animals to examine this problem further.

Although all of these studies provided interesting and valuable information concerning the various aspects of wound repair and inflammation, none of them was able to provide a definitive answer to the question, Can a fibroblast be derived from a hematogenous precursor? The value of parabiosis as an experimental tool to answer this question was demonstrated by Volkman and Gowans (29, 30). They were able to show that white blood cells serve as the precursors for the macrophages found in inflammatory exudates. The present study was similarly designed to attempt to answer the question in either a positive or a negative fashion.

The Protected Animal vs the Unprotected Animal

The protected animal was an effective control since it served to demonstrate whether or not those cells which were in the process of synthesizing DNA were capable of taking up thymidine as determined by light microscope radioautography. The radioautographs clearly show that essentially every cell type that would have been expected to be making DNA was, in fact, so occupied. This is substantiated by the observation that epithelial cells, endothelial cells, inflammatory cells, fibroblasts, and mast cells were labeled in large numbers in the wounds from the protected animals. The absence of label in epithelial, endothelial, and mast cells in the unprotected animals serves to demonstrate that little to no labeled thymidine crossed between the two animals at the time of the administration of the thymidine-^{3}H to the protected animal. It is presumed that the combination of clamping the cross-circulation and providing an excess dose of unlabeled thymidine at the time of injection of tritiated thymidine to the protected animal and at the time of release of the clamp served the purpose of diluting any small amount of label which may have crossed over from the protected to the unprotected animal.

It was possible by light microscopy to identify the large majority of the cells in the wounds of the unprotected animals as white blood cells. This left a small number of cells which, by light microscopy, could not be clearly identified as fibroblasts or monocytes.

It is easy to distinguish these two cell types with the electron microscope, largely on the basis of the abundance, distribution, and form taken by the rough endoplasmic reticulum in the fibroblast as compared with the mononuclear cell (Figs. 2 and 3). In addition, the mononuclear cell generally has a number of membrane-bounded, somewhat dense bodies distributed throughout its cytoplasm. The rough endoplasmic reticulum of the monocyte, although reasonably well developed and, presumably, at times associated with the synthesis of enzymes, is less well developed than that of the fibroblast and contains relatively large stretches of membrane devoid of ribosomes. Such stretches are unusual in the fibroblast (10, 25). In addition, the aggregates of ribosomes attached to the endoplasmic reticulum membranes of the fibroblast are large and contain as many as twenty to thirty ribosomes, whereas these aggregates in the monocytes are much smaller (25). With the use of these criteria, it could be seen by electron microscope radioautography that those labeled cells which could not clearly be identified by light microscopy were clearly identifiable as mononuclear cells with the electron microscope.

Although previously it was deemed possible that hematogenous white cells could have potentially become transformed, under appropriate stimuli, to connective tissue-forming cells, these studies clearly indicate that this is not the case in healing wounds. It must, therefore, be assumed that in instances of tissue repair in which connective tissue formation takes place, the fibroblasts come from a source other than circulating blood cells. These studies support the notion presented by Grillo (12–14), Glücksmann (11), Hadfield (15), and Dodd et al. (7) that wound fibroblasts are derived from cells in the connective tissue adjacent to the wound. Grillo (12) irradiated wounds locally 28 hr after wounding and demonstrated a 50% reduction in connective tissue cell proliferation. He could produce the same effect by irradiating wounds as early as 20 min after wounding when granulocytes represented the majority of the cells populating the wounds. Since neutrophils are short-lived cells and are unlikely to serve as a source of fibroblasts, it would appear that the mesenchymal cells which are located in areas adjacent to the wound and which were susceptible to irradiation were the source of fibroblasts. In addition, the cells which demonstrated the largest amount of uptake of thymidine by radioautography were the perivascular cells from the loose connective tissue in the wound margins.

Thus, the observation that no labeled fibroblasts were found in the series of studies where parabiosis was established, together with the observation of the uptake of thymidine after irradiation by perivascular connective tissue cells, clearly supports the notion that fibroblasts are not derived from blood cells but rather must come from the adjacent perivascular connective tissue.

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