ABSTRACT

Whether the fibroblast of granulation tissue is a product of resting connective tissue cells or is derived from circulating mononuclear blood cells is a question of dispute. At the same time it has been suggested that the small lymphocyte is capable of transformation into macrophages and fibroblasts. This investigation seeks to follow the behaviour of the lymphocyte in a surgical wound.

In vivo labelled thoracic duct lymphocytes are transfused into isologous rats and sections of surgical wounds at varying stages of healing are studied by autoradiography. In over four hundred and eighty-five slides examined, labelled cells were found in the wound on only two occasions, while it was possible to demonstrate labelled lymphocytes both in the circulation at 72 hours and in the alveolar septa at 5 days after infusion.

Transfused lymphocytes do not appear to have any significant structural role in the healing of fresh wounds, at least as can be demonstrated by the method employed.

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THE LYMPHOCYTE IN WOUND HEALING

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PREFACE

Research can be both challenging and stimulating, not to mention pedagogic. This project has provided some of each and has been a most rewarding experience.

I would like to acknowledge the assistance of Dr. B. Kopriwa of the Department of Anatomy, McGill University, both for technical advice and for preparing autoradiograms of some test slides; of Miss Jennifer Grove, University Surgical Clinic who prepared all of the slides for autoradiography, and of Mrs. H. Fernandez and Miss Pat Spicer for technical assistance.

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THE LYMPHOCYTE IN WOUND HEALING

INTRODUCTION

The role of the lymphocyte has caught the interest and stimulated the imagination of modern day researchers to an unprecedented extent. Investigators in the fields of immunology, morphology, and hematology have striven to unveil the mysteries of this cell; and while much is known about its cytology, phylogeny, ontogeny and distribution, there still exists divergent views as to its capabilities in immunology, differentiation and transformation. Indeed, there exists a school of thought which ascribes to it a pluripotential capability.

At the same time, the origin of the fibroblast in the healing wound remains somewhat of an enigma. There are those who believe them to derive from mononuclear blood cells, and others who would have them follow a connective tissue lineage.

This investigation seeks to follow the lymphocyte in a surgical wound and to determine whether they undergo transformation or modulation (52) and if they perform a structural role in the healing process.

HISTORICAL REVIEW

Studies with tritiated thymidine have led to the concept of a lymphocyte production pathway.[9,63] This begins with the primitive reticular cell after which a series of divisions occur, (eight according to Saint-Marie and Leblond)[56] and ends with the production of the lymphoblast. This cell then passes through the stages of the large and medium sized lymphocyte to its resting state of small mature lymphocyte.

Initially, it had been thought that this small lymphocyte represented an end stage, especially when it was shown that most small lymphocytes do not synthesise D.N.A.[7,13] This doctrine is no longer tenable today in light of the finding that the small lymphocyte can be stimulated to undergo blastogenesis by a variety of substances, both specific and non-specific. Phytohemagglutinin (P.H.A.), poke-weed mitogen (P.W.M.), Staphylococcal exotoxin and anti-human leucocyte antiserum are but a few of the non-specific agents, while tetanus toxoid, measles vaccine, tuberculin purified protein derivative and Streptolysin 0 are specific agents. The whole subject of transformation is well summarized by Daniels, Ritzman and Levin.[14]

Apart from antigenic stimulation, it is also believed that spontaneous transformation does occur.[37,55] Porter and Cooper employed both chromosomal and isotopic labelling to show that the transplanted small lymphocyte did indeed change into a larger cell type and subsequently went on to further proliferation.[49] Added to this is the proposition that the small lymphocyte can enlarge and once again become a reticular cell, thus holding the potential for the formation of two hundred and fifty-six new small lymphocytes.[56] Two ad-

ditional tenets have been proposed regarding the functional capabilities of the small lymphocyte.

Yoffey has advanced the thesis that lymphocytes exist in either conditioned or unconditioned states.[13] Conditioned small lymphocytes look exactly like their unconditioned counterpart, but have lost their primitive character and are incapable of developing along any other line than that of antibody formation. Most lymphocyte populations are probably heterogenous with a predominance of one or another cell type.

It has also been suggested that the small lymphocyte may be either active or inactive.[64] It can remain in the inactive state for rather long periods and this may explain the refractory response which has led many investigators to the conclusion that the small lymphocyte is an end-stage cell.

The blastoid cell which results from lymphocyte stimulation has been well studied and its morphology, biochemistry and relationship to other cells are described by many authors.[14] In addition, the claim is made for small lymphocyte transformation both through the blastoid cell and independent of it. Towards the end of the century many European authors were claiming that lymphocytes could and did transform into macrophages, at least in vitro.[14] After their description of the skin window technique, Rebuck and Crowley[51] went on to demonstrate the in vivo transformation of monocytes to macrophages.[52]

Large monocytes are also thought to sometimes develop from small lymphocytes. Bloom in 1938[4] suggested such a transformation and Fichtelius in 1957 by P32 tagging technique confirmed the idea.[39] In 1962 MacKinney used tritiated thymidine to prove the same phenomenon.

Besides claims for transformation to plasma cells, it has also been advocated that the small lymphocyte is a totipotential stem cell for hemopoiesis, at least in the marrow of the mouse. Transfusions of marrow or blood cells into lethally irradiated animals will sometimes initiate repopulation of the marrow and recovery.(25) Furthermore, it has been shown that granulocytes and monocytes are incapable of repopulating irradiated hemopoietic tissue(12) so that the new marrow must have derived from the totipotential lymphocyte.

That fibroblasts make some contribution to wound healing is generally accepted, with little understanding of the complexity and diversity of the mechanism. In addition to the biosynthesis of protein, polysaccharides and possibly even lipid, its physiologic functions include the providing of tensile strength through migration, orientation and differentiation; formation of fibrous tissue, cartilage and bone; wound contraction; remodelling and resorption.(15)

The protein collagen is made up of tropocollagen units which are arranged in an orderly fashion to produce the 640 A subperiods of the major repeating unit of about 2600 A.(40) Tropocollagen is itself made of fibrils which are specially arranged to conform to the characteristic banding of collagen. As the size of the fibril varies, so does the formed tissue - the smallest fibrils thus constituting embryonic collagen, the medium sized forming reticulin and the largest collagen.(62) Collagen as a protein is unique in having two amino acids - hydroxyproline and hydroxylysine, which are not found in other proteins to any extent.(1,61) Assay of hydroxyproline is a convenient measure of collagen production.(1) The mode of production is however disputed.(47,53,54,61)

The mucinous nature of ground substance has long been recognised. The acid polysaccharides responsible for this quality are either sulphated (chondroitin sulphate, heparitin or kerato sulphate) or non-sulphated (hyaluronic acid or chondroitin).[62] The manufacture of mucopolysaccharide by fibroblast both under normal and pathologic conditions is well described.[61]

In addition to the manufacture of ground substance, fibroblasts are believed by some to make lipids under special circumstances[15] and may also be involved in elastin production.[61]

Transparent window technique has shown the fibroblast to migrate and orientate itself along definite patterns[47,59,61] "like iron filings to a magnet".[47] Fibroplasia alone is said to contribute no more than 20% of the ultimate tensile strength, the remainder coming from collagen, and even more importantly through its "differentiation". This process is akin to ripening, and involves the aggregation of molecules by crosslinking through hydrogen bonds and stable covalent bonds. Differentiation is a function of stress and strain and for this reason scars mature faster after some activity.[15]

The origin of fibroblasts is from mesoderm along with blood cells, histiocytes, mast cells and others.[61] However, the origin of fibroblasts in the healing wound is often the subject for experimental and academic debate. One theory holds that such fibroblasts are derived from mononuclear blood cells and macrophages[1,2,22,29,32,34,36,41,44,49,50] while the opposing claim is for parentage from resting fibroblasts in connective tissue, the adventitia of small blood vessels and fatty tissue.[16,26,38,45,49,54,57,59]

In 1904, Maximow showed that the lymphocytes and monocytes of the blood wander into the tissues in inflammation and transform there into "polyblasts". He also conceded a percentage of these cells to derive from wandering cells or histiocytes. These findings were repeated in 1926 by the in vitro culture of guinea pig buffy coat cells.[41]

Polyblasts display an eccentric kidney shaped nucleus and a highly amoeboid cytoplasm with various inclusions. They may be as large as 12 microns or more in diameter.[40] They are either of histogenous or hematogenous origin.[40]

At the same time Bloom was culturing thoracic duct lymph of rabbits and observing large and small lymphocytes to change into typical inflammatory mononuclear cells or polyblasts, later converting to fibroblasts.[3] Carrel and Ebeling in supporting this work reported changes in cultures of large mononuclear cells to those morphologically resembling fibroblasts.[10,11] They considered the transformation to be an adaptive phenomenon of the monocyte to an unfavourable environment.

Moen cultured single monocytes collected from the thoracic cavity of rats reacting to intrapleural injections of low melting point wax.[44] By following isolated cells in the process of mitosis he described several types of fibroblast colony formation therefrom. Moreover, he states, "colonies of fibroblasts originating from mononuclear cells were easily transplanted, carried through repeated subcultures and still maintained their morphologic characteristics". This work, however, does not satisfactorily withstand criticism of purity from contamination by other cell types, and the method of identifying single cells was certainly not fool-proof.

That mononuclear blood cells are precursors of fibroblasts, at least in part, has also been supported by the work of Gillman,[29] Hartwell,[34] Hulliger[36] and Allgower.[2] The latter measured the hydroxyproline content of rabbit buffy coat cultures, collected by cardiac puncture, at varying periods up to forty-eight days and found an average of 14.4 $\pm$ 6.5 gamma for histologically transformed cultures as against 1.2 $\pm$ 0.9 gamma for non-transformed. In the control experiments there was a 4.6% and a 9.0% transformation for "heart" and "carotid" punctured buffy coat cultures respectively.[1] The actual amount of hydroxyproline in these cultures is not given, but as he concludes: "this would suggest that contamination could possibly account for one or two cultures per heart puncture, but could not yield up to thirty-seven cultures as observed in one of the experiments reported in this paper".

In 1961 Nicholas Petrakis demonstrated that buffy coat collected by venipuncture and cultured subcutaneously in volunteer subjects underwent definite regular changes. During the first two weeks, cells hypertrophied into polyblast macrophages and seven days later assumed the "stellate appearance or spindle shaped form of fibroblasts".[50] The conclusion was that "once polyblast macrophages are formed, they are able to metamorphose into fibroblasts, but they do not clarify the question of whether or not both lymphocytes and monocytes possess the ability to become polyblast macrophages, in vivo, or whether transformation is a property of only one of these mononuclear forms".

The experiments of Ross and Lillywhite cast doubt on the reliability of work employing venipuncture.[54] Buffy coat derived through cardiac puncture both with and without protective polyethylene tubing, and cultured in subcutaneously implanted diffusion chambers yielded 85% and 90% fibroblast and collagen

transformation respectively; that from the inferior vena cava and carotid artery showed only 10% and 6% change. Moreover, the authors doubt the authenticity of "fibroblasts" derived from cardiac puncture as electron microscopy reveals large deposits of glycogen and dense cortical filaments forming a continuous outer cytoplasmic layer.

Gillman injected tritiated thymidine into rats both pre and post wounding and then examined autoradiographs at varying periods post operation. He found 15-20% cells labelled in the post wound and 40-80% in the pre wound. Labelled cells were identified both intravascularly and perivascularly and cells resembling fibroblasts were labelled at varying stages of development. He believed that cells labelled in vivo migrate into injured tissue, become phagocytes and either leave the site or remain to undergo mitosis and become giant cells and fibroblasts.(27) In a follow-up study using similar techniques, he concluded that foreign body giant cells were derived from mononuclear blood cells.(28)

Fichtelius and Diderholm demonstrated greater numbers of labelled round cells and fibroblasts in the healing wound than in adjacent tissue. As there was no free thymidine available during healing (which started more than 72 hours after injection of thymidine) the increase in cells indicates an infusion from outside - "probably by nucleated blood cells". The level of label was, however, too low to allow any statistically significant appreciation of change, which could act as an indicator of re-utilisation.(23)

Evidence for the opposite ideology is given by Medawar, who demonstrated the evolution of polyblasts from lymphocytes only when the initial lymph specimens

were contaminated with numerous monocytes obtained via direct puncture of the thoracic duct.[42] This finding is confirmed by Hall and Furth who were unable to culture fibroblasts from carefully obtained thoracic duct lymph.[23]

On the other hand, Ebert presents evidence for the transformation of monocytes to histiocytes[16] but saw no change in small lymphocytes after 24 hours.[17] This latter finding is confirmed by Gowan after an extended period of five days although he did see divisions in large lymphocytes.[30]

MacDonald used tritiated thymidine and followed the developments in healing wounds from 2-14 hours after injections. His experiments demonstrated a rise in the label (70-120 hours) of fixed undifferentiated connective tissue cells along the adventitia of veins and arteries, amongst hair follicles and injured muscle fibres at a time when the number of labelled lymphocytes was decreasing (peak at 48-72 hours). On this basis he rejects the idea of lymphocytes transforming into fibroblasts.[33]

In a different approach to the subject, Peach studied regenerating tendon in the guinea pig by electron microscopy and laid emphasis on what he termed "migratory" versus "synthesising" fibroblasts.[47] The migratory type had elongated nuclei and were found at the advancing edge of the cellular sheet, while further removed from the centre and characterised by ovoid nuclei were the synthesising type. He summarises thus: "proliferating fibroblasts in the regenerating tendon appeared to be derived mainly from cells of the subcutaneous fatty reticulum and from the fascial sheaths surrounding the muscle bellies adjacent to the wound area".

The concept of different types of fibroblasts is also suggested by Stearns [59] and Edwards [21]. The former used the transparent window technique to follow the development of connective tissue in the healing wound. She observed fibroblasts to migrate inwards from pre-formed tissue peripherally. She described the invasion of the chamber by various blood cells and noted that although macrophages frequently assumed shapes resembling fibroblasts, this was only temporary and they did not transform into fibroblasts.

A different conclusion is reached by Ross [53]. It is his belief that macrophages are transitioned into fibroblasts. Superimposition of his graphs show similar trends for lymphocyte and fibroblast concentration in wounds. This is in variance with MacDonald who described fibroblastic proliferation at a time when lymphocyte concentration was decreasing [38].

Grillo x-irradiated guinea pigs both pre and post wounding and then examined, by autoradiography using tritiated thymidine, sections of these wounds at 5 days. Quantitatively, there was a 53% reduction in the cellular proliferation of wounds irradiated 28 hours after wounding; a 45% reduction on those 20 minutes post wound. There was no significant reduction in those which had been pre-irradiated. He concludes that irradiation at 28 hours depressed all cellular elements as did that at 20 minutes post wound, including the cells which became fibroblasts and those certainly could not be mononuclears as these are not present in the wound at the time. He dismisses as probably not significant the depression of capillary proliferation and admits that "some cells arriving from the vascular system either had or could develop the ability to function as fibroblasts" but summarises "these findings indicated that fibro-

blasts of wound repair are predominantly connective tissue cells." (32)

The ability of bone marrow cells to stimulate a cellular reaction in irradiated rats given Freud's adjuvant was demonstrated by Spector and Willoughby.(58) At the same time, animals so treated and receiving either lymphoid or thymus cells showed a similar response to those receiving no infusion - necrotic and degenerate polymorphs at 3 days, eventually showing good mononuclear response at 14 days as the marrow recovers. When tritiated thymidine is used an an index of mitotic activity a similar pattern is seen. This data led to the conclusion that "normal rats bone marrow cells are the original source of macrophages, histiocytes, perithelial cells and small round cells that abound in chronic inflammatory granulomata".

These arguments are but a capsule presentation of the many papers published. Strong criticism can be made against many of the investigations quoted on the basis of purity of cell type employed: strength and indelibility of label: transformation versus modulation: definition and indentification of fibroblasts, but even if such criticism be justified, it in no way answers the basic question of the derivation of fibroblasts in the healing wound.

On such a basis, a protocol was designed to follow the course of the lymphocyte in wound healing without prejudice to either opinion. If in vivo labelled lymphocytes could be collected by thoracic duct drainage, maintained in a viable state, and injected into isologous rats, then it should be possible to follow their distribution and behaviour by autoradiography.

MATERIALS AND METHODS

ANIMALS: Highly imbred strains of Lewis rats were used (LEW/f Mai) in weights of 50-100 gms. These are isohistogenic. Animals of either sex were used, but donors and recipients were always matched. Standard diets were employed but during thoracic duct drainage the rats were fed 0.45% NaCl orally.

CAGES: During drainage rats were restrained in conventional wire cages, a section of which was modified in the manner of the Bollman cage [5] (see Fig. 1). This allows the animal to move back and forth without being able to turn around. Water and food are freely accessible at the front of the cage. A horizontal free area at the side allows the catheter to slide with the animal's movement (Figs. 1 and 4). A collecting device was attached outside and somewhat lower than the cage (Fig. 1).

A costly problem of rats revolving around their long axis and thus entwining themselves in the catheter or displacing it from the thoracic duct was finally overcome by employing a body jacket of adhesive tape. This jacket is attached above to a sliding rod which moves with the animal but prevents it from spinning (Figs. 1 and 4). Heat is provided by a 100 watt bulb at controlled distance.

THORACIC DUCT LYMPHOCYTES: The thoracic duct was connected by a modification of the method described by Bollman et al. [6] Ether replaced intraperitoneal nembutal for anaesthesia as it is essential to have the animals awake and active soon after surgery if proper drainage without clotting is to be obtained. The rats were not heparinised. The method adapted allowed for varying the distance between the ether source and the animal, thus being able to control the

the air-ether mixture being absorbed.

The abdomen was entered through a full length midline incision and the aorta was identified between the fibres of the psoas muscle. The thoracic duct was found closely attached to the aorta in the left posterior position between the diaphragm and kidney. It was carefully dissected off the aorta in its upper section as to work in the lower aspect may lead to rupture of any of the many radicals feeding into the cysterna. After the duct is ligated (5"0" black silk), it dilates distally and becomes easily visible. Too much tension in the system makes for easy rupture.

Catheter tips are made of polyethylene tubing (1.D 0.030") drawn out to a fine point in heat. This is joined to a silastic tubing of approximately 3 cms. and at the far end the remainder of the catheter is made up of polyethylene. Where the silastic is joined to the polyethylene, two flanges are made to hold sutures (Figs. 2 and 3).

After the duct is canulated, a second tie is placed around it to hold the canula and the initial tie is secondarily secured around the catheter to hold it in place (Fig. 2). In addition to this, one flange is sutured down to the closely applied psoas muscle (Fig. 2). The silastic readily takes the curve of the diaphragm and the other flange is sutured to the body wall in the flank through which the catheter is exteriorised (Figs. 3 and 4).

The abdomen is then closed in layers (4"0" silk). The catheter is not siliconised but is filled with a solution of heparin and Krebs' Ringer buffer at the time of canulation.

COLLECTION AND PROCESSINE: Lymph is collected at room temperature in a flask containing 3-5 ccs. Krebs' Ringer Solution, 40-60 units of heparin and 150 ug Streptomycin. The flow of lymph can be regulated by the strength of NaCl fed to the animal [26] but without increasing the output of cells.

Eight to ten hour specimens are collected and centrifuged at 120 g. for 10 minutes. A suspension is then made with fresh Krebs' Ringer Solution and a count is made of the cell concentration (Hawksley haemaccytometer). A viability study is made by eosin staining and a smear is taken for autoradiography. The suspension is then injected into the recipient animal by tail vein.

Tritiated thymidine is used as a label (Schwarz Spec. Act. 10.0 C/mm. aqueous). Rats are injected intraperitonealy on a schedule of 14 daily doses of 0.5 uc/gm body weight and catheterised 2-3 days after the last injection.

Standard surgical wounds are made through all layers of the abdomen of the recipient rats, 3 cms. in length. Injections of labelled cells are made at varying times after wounding and sections of the wound are taken at different periods after the last injection.

AUTORADIOGRAPHY: Sections are fixed in 10% formal saline for 24-36 hrs. and then prepared by ordinary histological technique. They are cut 6 u thick, mounted, and the slides labelled.

The dipping procedure as outlined by Messier and Leblond [43] is followed using Kodak nuclear track emulsion N2B2. Dipped slides are stored at $4^{o}C$ in a light exclusive black plastic box containing 15-20 gms. of indicating drierite.

The optimal duration of exposure is determined by serial development of test slides. This is usually 3-4 weeks.

Slides are developed in freshly prepared Dektol for 6 minutes, rinsed in distilled water for 1 minute and fixed in sodium thiosulphate for 3 minutes. They are then rinsed in running tap water for 8-10 minutes. Post-staining is with hematoxylin and eosin (H & E).

CONTROL: Three types of control sections are taken. The first is of an ordinary surgical wound for conventional histology to establish the pattern of repair. For this, sections are made at 6 hours post wound and every 2 hours thereafter to 24 hours; at 36, 72 and 120 hours. They are stained with H & E. The second is taken from a labelled animal which has undergone all stages up to dissection of the thoracic duct. The abdomen is then closed and sections made at 24, 72 and 120 hours. This procedure is termed "Sham catheterisation". The third type follows the regular protocol but cells are killed by heating in a water bath at 45°C for 10 minutes.

RESULTS

Figs. 5-9 outline the cellular changes in a surgical wound. Definite leucocyte infiltration is evident as early as 6 hours post wounding (Fig. 5) and while this continues to increase throughout the first day (Fig. 6), there is not a substantial gain over that seen at 6 hours. Cellular alignment is present as early as 48 hours and quite obvious by 72 hours (Fig. 7). At 5 days there is an orderly arrangement of all cellular components (Figs. 8, 9).

Cells resembling fibroblasts begin to make their appearance around 36 hours at which time they are fairly large (10-12 u) with a nucleus occupying most of the cell. The cytoplasmic processes are stunted. By 48 hours, they have become more spindle shaped with either central or polar nuclei, having both more and better developed processes. Those seen at 72 hours are fully developed fibroblasts (Fig. 7).

Figs. 10 and 11 represent the only sections in which labelled cells were found in the wound. As such, they were taken from two different schedules. The animal of Fig. 10 received a transfusion of 64×10^6 cells in two divided doses at 2 and 8 hours after wounding. These were harvested from a rat some 16 hours after the last injection had been given and yielded a slightly higher percentage of labelled cells (33%). As well, the autoradiogram here was exposed for 32 days and sections were taken at 96 hours after wounding.

Fig. 11 represents an animal which received a total of 1008×10^6 cells given over a period of three and one half hours. These cells were taken from a pool of lymph collected from 3 rats injected on the regular schedule. The

exposure time was 25 days and sections were made at 30 hours after wounding. This section shows a labelled cell in the deep layers of the dermis.

Figs. 12 and 13 are of lung and were taken from the same animal as in Fig. 11 but at 120 days after wounding. Several well labelled cells are shown in the alveolar septa.

Another section of lung is shown in Fig. 14. This is taken from an animal receiving 360 x 10^6 cells from a donor treated on the routine schedule. The cells were injected at the time of wounding and while no labelled cells could be found in the skin, liver or nodes, there were several in the lung and in the pulmonary circulation.

A section from an intestinal lymph node is shown in Fig. 15. This tissue is taken from the same animal as in Figs. 11, 12 and 13 at 120 hours after wounding. It represents the only time a labelled cell was found in a lymph node although the search was made in 3 animals over 15 separate sections.

The remaining photographs were taken of control animals. Figs. 16-19 inclusive are taken from the same rat. This animal received the regular dose of tritiated thymidine and was then subjected to laparotomy and thoracic duct dissection but without canulation. A section of the abdominal incision was taken at 24 hours.

Fig. 16 shows a strong label in many cells even between muscle fibres. In Fig. 17 is seen connective tissue in which the greatest majority of cells are well labelled. This section is taken from an area near the incision and

subjacent to the epidermis. Fig. 18 is a high power view of a labelled cell deep in the dermis. Fig. 19 shows the label in the cells around the hair follicles.

Fig. 20 is taken from an animal on the same routine as that in Figs. 16-19, but in which the section was made at 5 days. Here cellular label is significantly reduced (compare with Fig. 18).

The last section represents liver and is taken from an animal receiving 216×10^6 cells on the routine schedule. However, in this case the cells were killed by heating ($45^{\circ}C \times 10$ minutes) prior to infusion. Sections of lung, skin and node revealed no labelled cells and only in one slide was a labelled cell seen in the liver (Fig. 21).

DISCUSSION

The techniques adapted in this protocol sought to overcome the difficulties inherent in the experimentation of cellular patterns and behaviour.

Methods are available for the differential separation of white cells, but the yield of viable uncontaminated cells is low (20-30%). Thoracic duct lymph is probably the richest source of the least contaminated lymphocytes readily available for experimentation. It is of course essential in an investigation of this nature to use a suspension of cells as free as possible of contamination by other cell types, especially where such other cells may also bear the label being employed. For this reason, thymic lymphocytes were not used. Rat buffy coat has too small a yield to be of value.

Indelibility of label is the objective in all experimentation where markers are employed and the method which is closest to this ideal is isotopic labelling. Tritiated thymidine is incorporated into the D.N.A. of actively dividing cells just prior to mitosis and remains there as long as the cell lives, being halved at each division.[19,35,57] As a nuclear label, tritium presents advantages in autoradiography because of its very high resolution obtained through very weak energy of only 17.9 Kev. and a short B radiation of less than 6 u. Actually, half of the B particles will travel less than 1 u.[24] Its half life is 12.1 ± 0.5 years.[46] Its apparent availability to cells for approximately only 45 minutes means certain specific physiologic states can be marked.

There are some investigators who question the stability of the D.N.A. label[48] and others who wonder about re-utilisation when cells die.[8,60] It is

not possible to either prove or disprove re-utilisation, but on theoretical grounds it would only be a real consideration where the tissue being studied had either a high rate of cellular death or cellular turn-over. In either case the label would have to be very strong if it is not to be diluted beyond the point where it would no longer be demonstrable by ordinary exposure time and development technique.

When cells change, there are two patterns along which they develop. Cellular transformation in which the morphological changes do not obliterate the original characteristics is termed "modulation". It is a reversible functional adaptation. Cellular "differentiation" on the other hand occurs when a differentiated cell acquires characteristics foreign to it and loses some of its own original properties.[52] Rebuck believes the small lymphocyte to undergo modulation.

More than a little of the difficulty encountered in the understanding of the origin of fibroblasts in the healing wound is a result of lack of definition. To most histologists, the fibroblast is a stellate, triangular or drawn-out cell with an ovoid nucleus containing dust-like chromatin particles and one or more large nucleoli.[40] However, Stearns visualised fibroblasts to have a changeable morphology. If cells resembling typical fibroblasts were stimulated, they were seen to roll up in circular form and remain quiescent. After a period they would once more assume the shape of a fibroblast.[59] As well, they have been described as active and inactive, synthesising and migratory.[47]

It is hardly surprising therefore to understand why the earlier investigators who used only fixed histological technique, should be of widely divergent

opinion. Neither is it difficult to see the confusion which can arise when cells are poorly marked. One of the more reliable techniques as far as the fibroblast is concerned makes use of hydroxyproline measurement as an indicator of cell activity and presumably concentration.[1] Where both chromosomal and isotopic labelling are used, no valid argument can arise as to the indefectible identification.

While it is possible to obtain a better percentage label, the scheme adapted here yields a label in 28-30% small lymphocytes.[18] The longer the period between catheterisation and last injection, the fewer labelled medium and large sized lymphocytes will be harvested. This may be a small factor in explaining the positive result in Fig. 10, where this interval was shorter than usual.

The only other positive result among 485 autoradiograms is represented in Fig. 11. This is also the only animal to receive more than 360 x 10^6 cells. As such, it received a transfusion of 1008 x 10^6 cells in 3.5 hours, a value equal to its total body small lymphocyte composition and representing some 33.3-43.8 times its circulating small lymphocyte population.[20] It is inconceivable that labelled cells be not found in the healing wound of such an animal. Chance alone would dictate it. What is indeed surprising is that only one such labelled-cell has been found from a total examination of 25 autoradiograms of skin tissue in this rat.

It may be theorised that early injections or indeed continuous infusion may have yielded positive results especially in light of the finding in Fig. 10. While this might be true, it must be pointed out that the exposure in Fig. 10 is longer than usual, thus partly explaining the higher background count. This sup-

position in no way explains the number of labelled cells found in the lung of the same animal at times up to 120 hours after wounding. The same argument may be given for the evidence in Fig. 14 which shows labelled cells circulating (or recirculating). Fig. 15 is another single example of a labelled cell, this time in a lymph node. It represents the same animal as in Figs. 11, 12 and 13.

The cellular outlines in Fig. 10 and 11 are not well delineated, but on light microscopy both labelled cells resemble fibroblasts. While this is not true in Figs. 12-15 it does not categorically bespeak differentiation or modulation. However, when compared with autoradiograms of the injected suspension, there is little or no reduction in the grain count, thus negating the possibility of re-utilisation. This argument is strengthened when Figs. 14 and 20 are compared.

A control element is provided in Figs. 16-21. The first four are of labelled cells in the injected animal to show strength of label and grain count. On these two criteria, Figs. 10-15 compare admirably. A 5 day old section is shown in Fig. 20 and the grain count is markedly reduced when compared to the suspension, and even to the preparation of transfused cells of the same age as shown in Figs. 12, 13 and 15. There is no straight forward explanation for this phenomenon.

The finding of a labelled cell in the interlobular area of the liver (Fig. 21) is difficult to explain. Both in the photograph and on light microscopy it is difficult to identify the cell type, although the shape is more sug-

gestive of fibroblast than lymphocyte.

It is apparent that some cells become trapped in the lung (Fig. 12-14), but the numbers seen can hardly account for the total number of cells injected. It would indeed be interesting to know the ultimate fate of such cells, as they do not accumulate in the wound and are not to be found in the liver or nodes to any significant degree. It is unreasonable to suggest disintegration as there was no evidence of label or debris in the reticulo-endothelial system studied.

The methodology involved in this experiment is such that a period of seven to eight weeks must elapse before any results are obtained. While one may speculate and vary the several parameters in the design, definite change can be made only when autoradiograms are studied. To further complicate the situation, there are several points at which mistakes can very easily be made, not the least of which is catheterisation of the thoracic duct and being able to establish a good duct flow. Proficiency in these techniques requires much patience and practise and was responsible for a good deal of the time involved in this work. This circumstance was aggravated by the fact that the methods described in the literature were unsuccessful in my hands. Another point causing delay was the difficulty experienced in restraining the animals, and several modifications had to be made to techniques described by others.

Despite the handicaps described, a total of 485 sections were studied in 18 rats receiving transfusions and as already indicated labelled cells were identified in the wound on only two occasions. While some autoradiograms may be criticised as having too strong a background, this defect is more likely to lead to

false positive results than vice versa. The sensitivity of the technique is delicate enough to locate labelled cells in the lung after 5 days and can hardly therefore be advanced as an explanation for the lack of positive findings.

No good explanation is forthcoming from this study to explain what happens to the transfused cells, but as this was not the intent of this protocol, no detailed attempt was made to locate them, except in the sections of lung, liver and node as indicated. However, several possibilities exist. Murray and Woods have suggested that thymic lymphocytes appear to home for spleen and mesenteric nodes as well as the gut lumen through the mucosa.[45] It has also been shown by Everett,[31] and supported by others that there is a considerable degree of recirculation between lymph and blood, and in fact, transfused labelled cells may account for as much as 80% of the increase seen in thoracic duct lymph after such transfusions.[31] Perhaps some or all of these factors may be responsible.

CONCLUSION

It is evident from the data that injected small lymphocytes do not take any significantly active part in the healing of acute wounds, at least as can be demonstrated by the method employed.

When a number of lymphocytes comparable to an animal's total body composition is injected, several labelled cells are found in the lung up to 5 days after injection, but on only one occasion was there a labelled cell in the wound.

If fewer than this number are injected, positive autoradiograms are found on only one occasion from a total of 485 slides; and this is seen in an animal which had received transfusion at the time of wounding. It may well be that this is related to the rather early infusion of white cells into the wound (Fig. 5), a fact that is not generally appreciated.

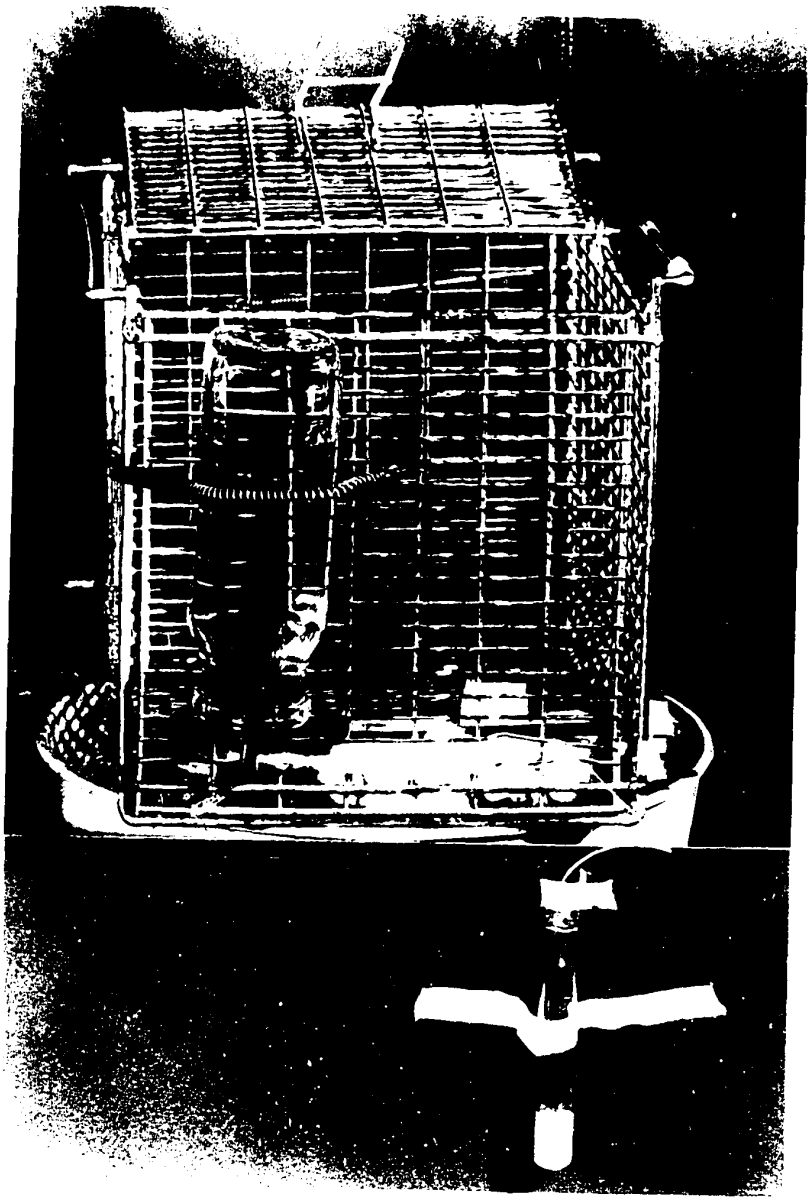

Fig. 2 Canula in the thoracic duct with extra tie from flange to psoas muscle. The aorta lies on the medial side of the duct.

Fig. 3 Canulated thoracic duct. Additional fixation is achieved by suturing a flange to the body wall.

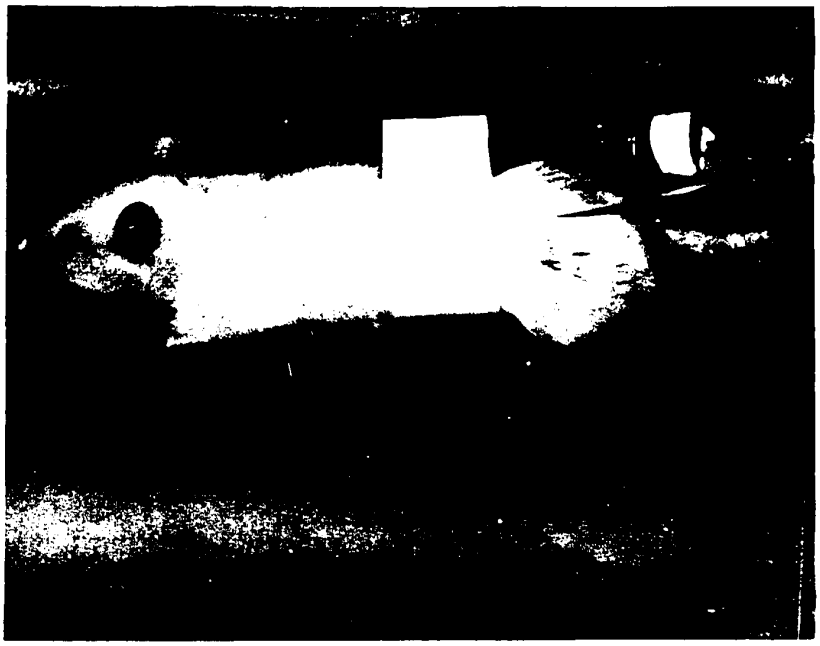

Fig. 4 Canulated rat draining lymph. A body jacket is in place and exteriorisation of the duct through the flank is shown.

Fig. 5 Surgical wound of skin at 6 hours, showing early infiltration by white cells. (x 40)

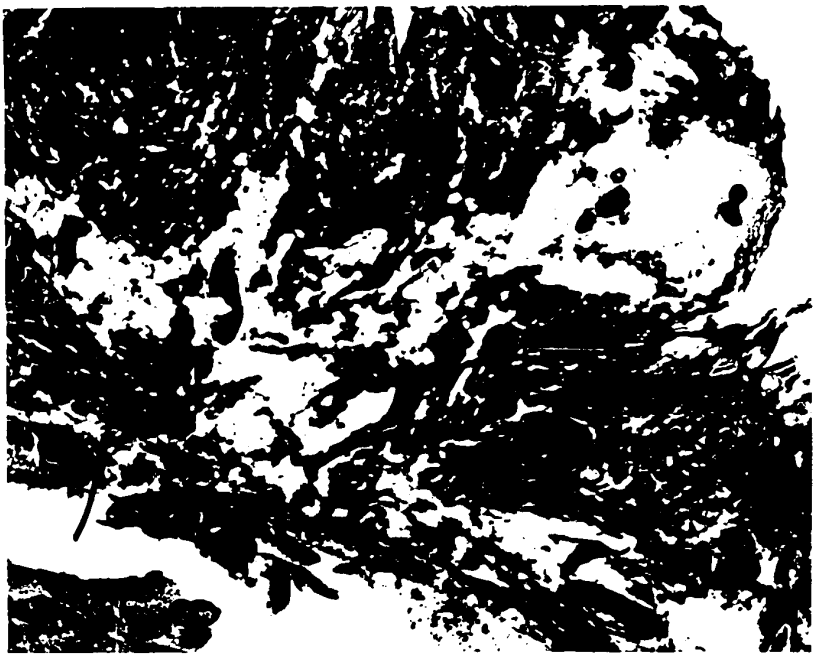

Fig. 6 Surgical skin wound at 18 hours. There is minimal increase in the numbers of inflammatory cells. (x 40)

Fig. 7 Surgical wound of skin at 72 hours. Fibroblastic proliferation and orientation are well demonstrated in all areas. (x 40)

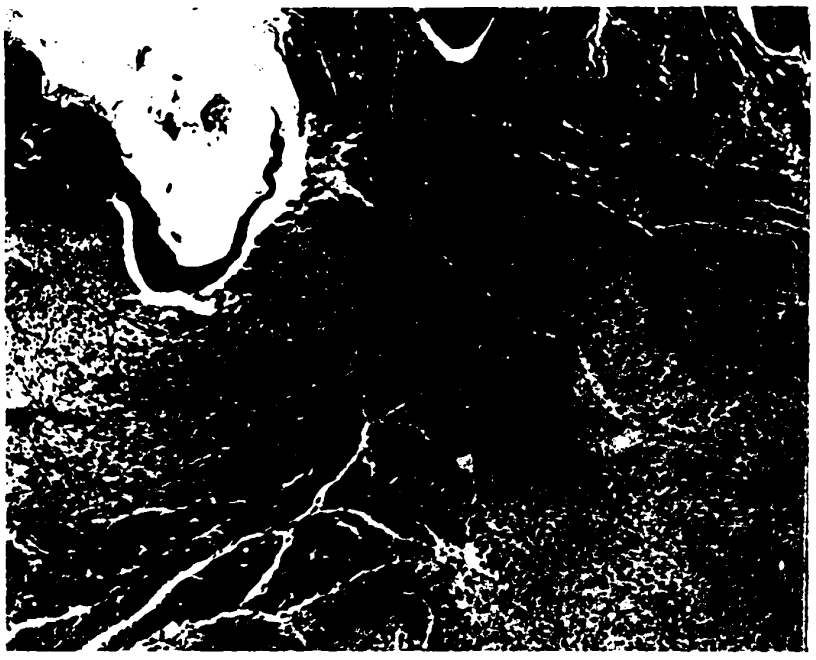

Fig. 8 Surgical wound of skin at 120 hours. Note the degree of orientation and differentiation. (x 40)

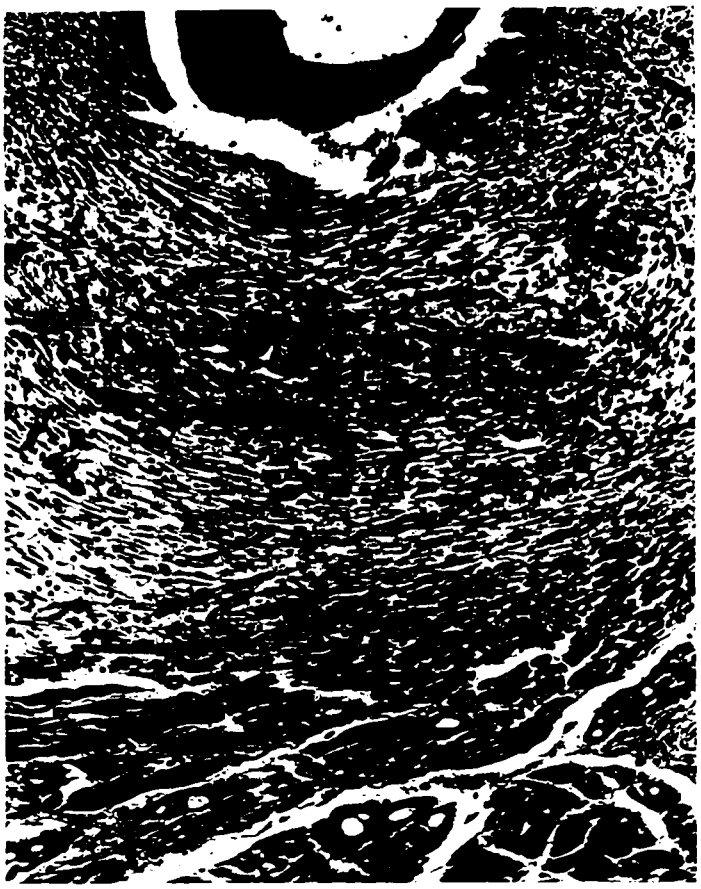

Fig. 9 Surgical wound of skin at 120 hours. Cells are mature fibroblasts arranged along definite lines. Most of the inflammatory debris has been cleared. (x 100)

Fig. 10 Autoradiogram of skin wound at 96 hours. After wounding showing single labelled cell and heavy background. (x 1000)

Fig. 11 30 hour post wound autoradiogram of rat receiving 1008 x 10^6 labelled cells. Area represents deep dermis. Note single labelled cell on the surface. (x 1000)

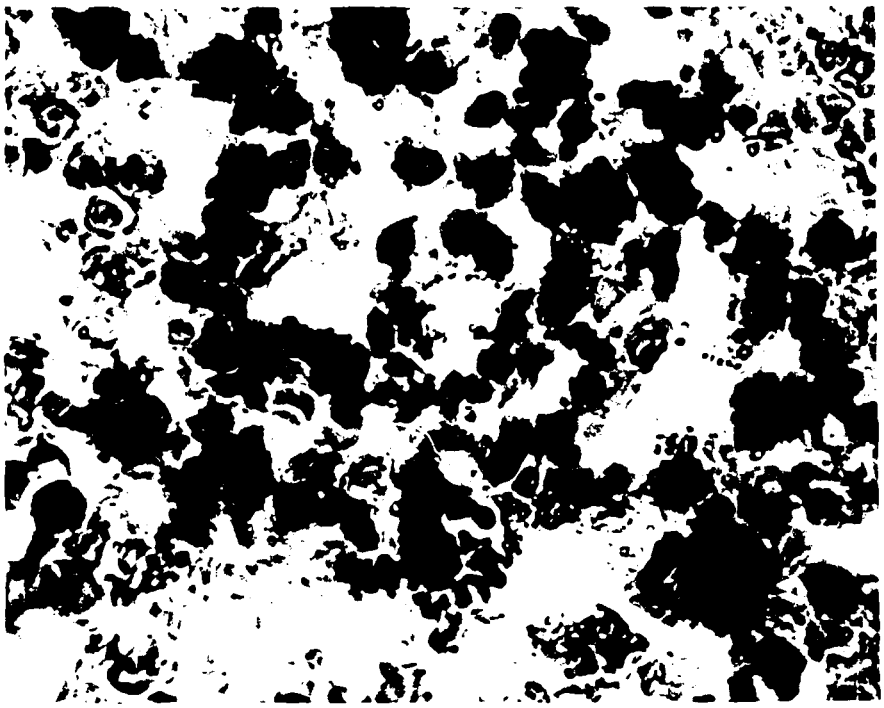

Fig. 12 Section of lung at 120 hours post wound. There are several heavily labelled cells in the alveolar septa. (x 250)

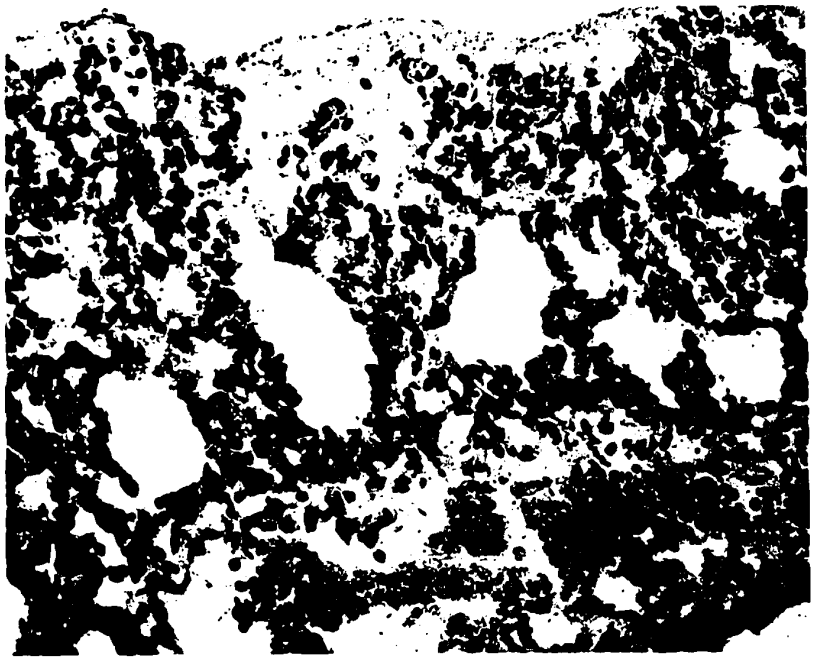

Fig. 13 A section of lung from the same animal as Fig. 12. A low power view showing the spread of labelled cells. A large vein is seen inferiorly. (x 250)

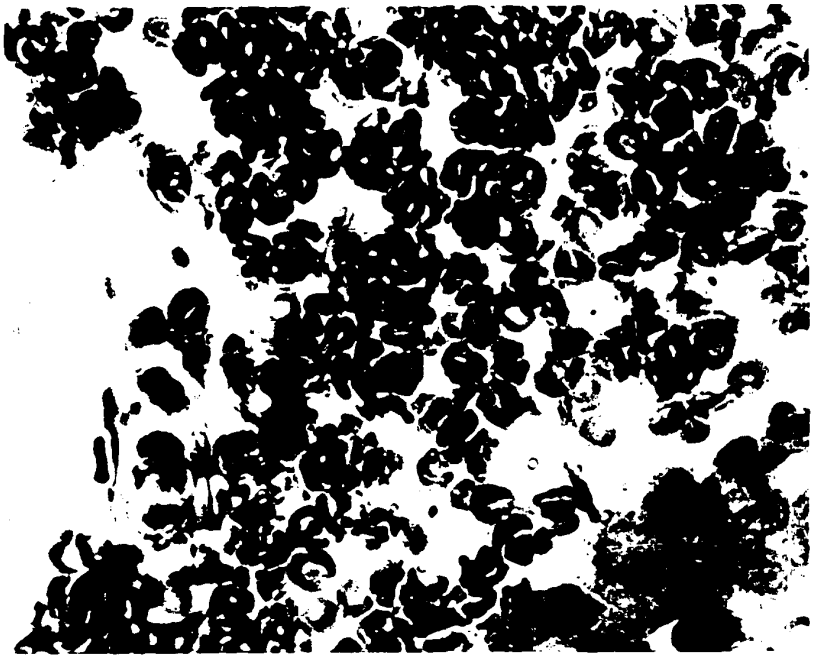

Fig. 14 A 72 hour post wound autoradiogram of lung from an animal receiving 360×10^6 cells. Three heavily labelled cells are seen in a vein. (x 1000)

Fig. 15 Section of the lymph node at 120 hours post wound showing single labelled cell. Taken from the same animal as Figs. 12 and 13 (× 1000)

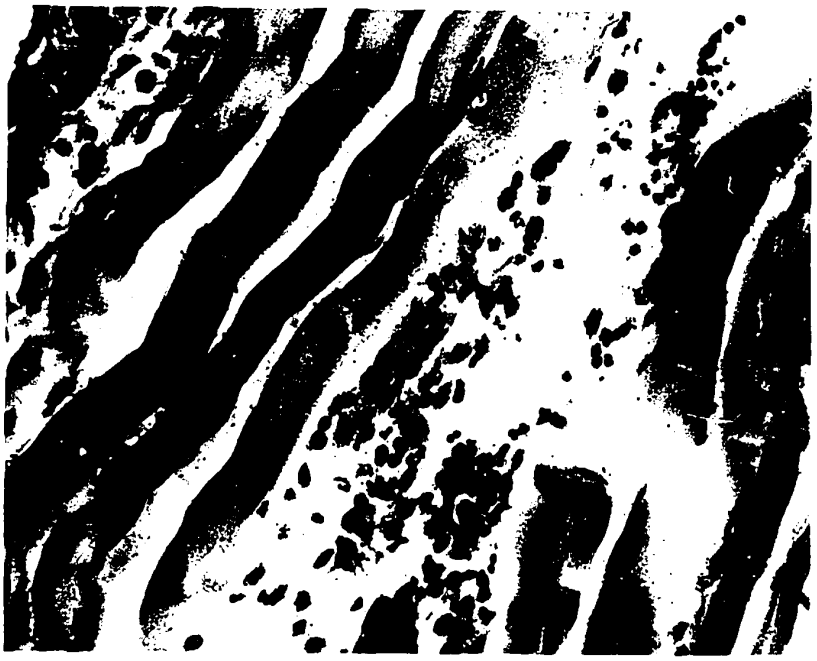

Fig. 16 24 hour post wound section from a labelled rat having sham catheterisation. There is a strong label in most fibroblasts including those among muscle fibres. (x 250)

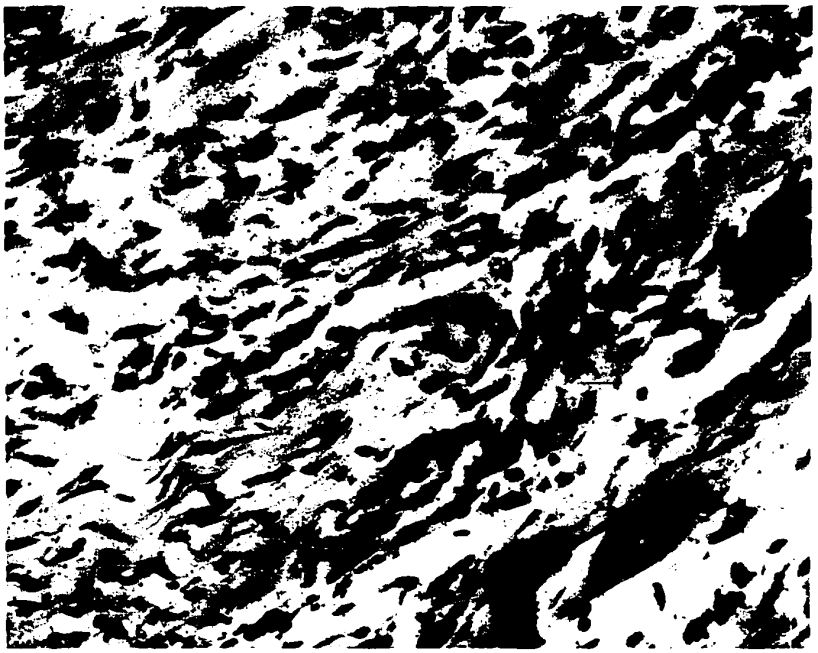

Fig. 17 Granulation tissue from the skin of the same animal as in Fig. 16. There are high grain counts in most labelled cells. (x 250)

Fig. 18 High power view of labelled cells in the skin of a rat which had received the normal schedule of isotopic label, but not catheterised. (x 1000)

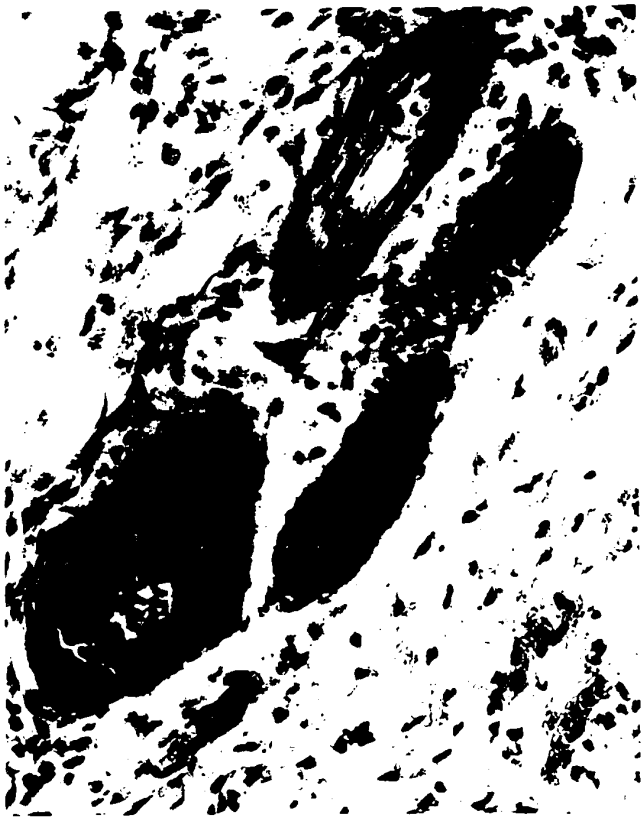

Fig. 19 Section from a labelled rat sham catheterised showing the degree of label in cells around hair follicles. (x 250)

Fig. 20 This control section demonstrates the degree of dilution in labelled cells at 120 hours post wound. Taken from a labelled rat sham catheterised. (x 1000)

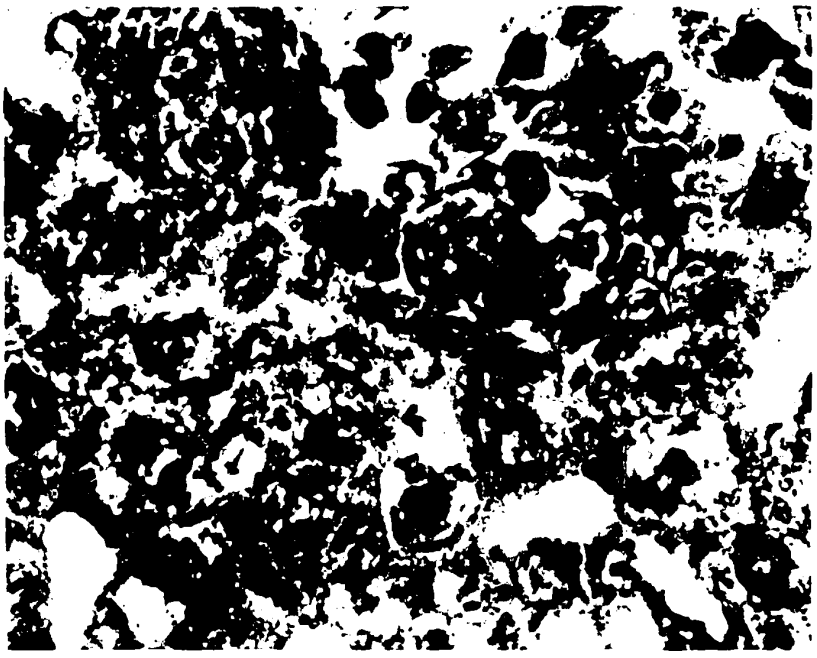

Fig. 21 At 72 hours post wound two labelled cells are seen in the liver of a rat receiving a transfusion of dead labelled cells. (x 1000)

Time of section after wounding (hrs.)	2	9	20	24	30	36	48	72	96	120	168
Nos. of rats	1	1	1	5	2	1	3	9	2	13	7

TABLE I

Time of section after infusion (hrs.)	2-3	12-14	18-20	26-30	40-50	60-70	70-80	80-90	90-100	100-120	120-140	140-160
Nos. of rats	3	4	2	3	3	8	3	2	3	10	1	6

TABLE II

No. of cells injected (10^6)	0-20	20-30	30-40	40-50	50-60	60-70	180-185	216	360	1008
Nos. of rats	1	3	1	3	2	2	2	1	1	1

TABLE III

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