A RADIOAUTOGRAPHIC STUDY OF LYMPHOCYTE PROTEIN PRODUCTION IN SKIN ALLOGRAFT REJECTION

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DALE CORWIN PEERBOLTE

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This is to certify that the

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ABSTRACT

A RADIOAUTOGRAPHIC STUDY OF
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By

Dale Corwin Peerbolte

In an attempt to demonstrate that lymphocytes produce proteins within allograft beds, and also to make observations on the fate of those proteins, analyses were performed on 64 skin allografts and autografts placed on ten C^3H and A/J strain mice. Tritiated thymidine given 24 hours prior to the grafting labeled the nuclei of blood lymphocytes and made possible their subsequent identification in the graft beds. Four days after grafting, when lymphocytes had entered allograft beds and begun to hypertrophy, tritiated leucine (H^3L) was given to the animal to label the protein being produced. Allografts, autografts, and the surrounding tissues were analyzed at various time intervals ranging from one hour to six days following the H^3L injection.

The thymidine label, which localized mainly over nuclei, made it possible to identify hypertrophied lymphocytes in allograft beds, even though reutilization of the label was shown to have occurred. By comparing the leucine label in allografts to that seen in the fibroblasts of

autografts, it was observed that a labeled material (presumably protein) was produced in the cytoplasm of hypertrophied lymphocytes in allograft beds and subsequently was slowly released from the cells.

In the graft tissue above the beds, allografts were continually accumulating protein (with a H^3L label), while in autografts a decrease in the radioactivity indicated a normal protein turnover rate.

It was concluded that the lymphocyte release of radioactive material (presumably protein), demonstrated in allograft beds, could account for the majority of the radioactive accumulation in allografted tissue, and therefore was a major factor in the akin allograft rejection reaction.

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INTRODUCTION

Immunology is one of the more important areas of medical practice today, for in its realm lie a myriad of diseases which have plagued man for centuries. Immunology basically involves the capability of the body to recognize and destroy a foreign substance which may cause it harm. This broad field has been divided into two general areas, namely, (1) humoral immunity, which involves such phenomena as antibody production and antigen-antibody interaction; and (2) cellular immunity, which involves sensitized cells, cell-to-cell interaction, and cell-bound antibody.

Cellular immunity seems to function by guarding the body against foreign tissue grafts and certain forms of malignancy. These two have led to a dichotomy in cellular immunity research. In tissue grafts, the reaction is undesirable and needs to be inhibited if the graft is to survive, whereas in malignancy, the reaction is desirable, and needs to be enhanced so that malignancy can be eliminated. Nevertheless, in both areas it is necessary to understand the basic mechanisms operative in the rejection reaction; so that, provided with sufficient knowledge and ability to control the processes involved, treatment of such ailments as cancer and terminal heart or kidney diseases could be greatly facilitated.

One method for studying the graft rejection reaction which has been widely used is the skin allograft system. It serves as an adequate model for the process because the features of lymphoid cell infiltration, blast transformation, and foreign cell destruction are similar to those occurring in other foreign tissue transplants, and can be easily observed, manipulated, and quantified.

The present experiment seeks to clarify one aspect of the allograft rejection reaction, namely the role of hypertrophied lymphocytes of the graft bed in the reaction against allografted skin in mice. More specifically, the question is how these hypertrophied lymphocytes and their products participate in the death of the foreign cells in skin allografts in vivo, and whether this parallels what they seem to do in allogeneic systems in vitro.

Objectives for the present study were:

(1) to identify hypertrophied lymphocytes in the graft beds.

(2) to identify proteins being produced by cells within the graft bed.

(3) to trace these proteins and analyze their possible role in graft rejection.

LITERATURE REVIEW

The amount of research literature which has accumulated over the past years regarding the graft rejection reaction makes it inappropriate to attempt to review all of the related information available. Rather, the present review will select appropriate orginal research articles and pertinent recent reviews which are representative of many others and which are directly applicable to this study.

In skin allograft rejection a series of events takes place which begins shortly after the grafted tissue is placed, and which culminates in death of the foreign cells and their elimination from the body. Known events in the reaction include vascularization of the tissue; infiltration by lymphocytes, monocytes, and polymorphonuclear cells (Feldman, 1969); hypertrophy of the invading lymphocytes with accumulation of basophilic cytoplasm (Jakobisiak, 1971 and Walker and Goldman, 1963); followed by the progressive destruction of the grafted cells and the vascular tree (Feldman, 1969, Lykke and Cummings, 1970, and Liem and Jerusalem, 1970).

It is the origin and role of the infiltrating lymphocytes which has been the focus of much attention. The immediate origin of the cells is the blood stream of the host.

Using tritiated thymidine to radioactively label short-lived cells, Walker and Goldman (1963) found that the radioactive cells in the graft bed were similar in labeling characteristics to the large lymphocytes of the peripheral circulation. Griffiths (1970) also labeled short-lived lymphocytes with tritiated thymidine and found the cells invading allografts in large numbers. These cells are thought to be of bone marrow origin (Osmond, 1969), which, under the influence of the thymus are rendered capable of recognizing antigen (Basten et al, 1971 and Yunis et al. 1971). These thymus-dependant lymphocytes (T-cells) are distributed throughout the lymphatic system and peripheral circulation (Miller et al. 1971_a) and, upon contact with a foreign graft, migrate to the draining lymph nodes (Tilney and Gowans, 1971 and Zatz and Lance, 1971). Here (or in the spleen) the antigen stimulated cells are thought to interact with effector cells (Giroud et al. 1970, Miller et al. 1971_b, and Lonai and Feldman, 1971_a) which are then released by way of the thoracic duct (Chanana et al. 1969) where they rejoin the circulation.

The nature of the antigen-sensitive and effector cell interaction has generated much interest and speculation. Much of the research involves in vitro models of rejection systems to study the various cell populations involved. T-cells, B-cells (non-thymus dependant), and macrophages interact in the presence of antigen and produce a cell type which

is capable of destroying target cells which have the sensitizing antigen. Whether T-cells render B-cells cytotoxic (Grant et al. 1972, Billingham, 1969, and Miller et al. 1971_b), or whether the T-cells themselves are the effectors (Miller et al. 1971_c, Golstein et al. 1972, Cerottini et al. 1970, and Wagner et al. 1972) or whether macrophages are the effectors (Salvin et al. 1971, Kramer and Granger, 1972, and Evans and Alexander, 1971) remains to be determined. However, it has been found that all three are required in an interaction to produce the optimal response in vitro (Feldmann, 1972, Gisler and Dukor, 1972, and Lonai and Feldman, 1971_b).

Once these activated effector cells have re-entered the circulation, they infiltrate the graft area (Goren et al. 1972) and destroy the foreign cells. The mechanism of this destruction involves cell-mediated cytotoxicity which is even less well understood than the interaction at the lymph nodes. Controversy exists as to whether the action is immunospecific or nonspecific (Moller and Lapp, 1969 and Hayry and Defendi, 1970) and whether or not it requires direct cell contact (Benacerraf and Green, 1969). The most plausible explanation at this time is that there appear to be at least two processes by which effectors can destroy foreign tissue. One process involves direct immunospecific action in which cell-to-cell contact is mandatory, and the other process involves a nonimmunospecific cytopathic effect, which also acts as an inflammatory agent (Billingham, 1969).

In vitro cell mediated cytotoxicity can be detected by Cr^{51} release from susceptible target cells (Peter and Feldman, 1972). This cytotoxic activity was found to be parallel to the time sequence noted for rat skin allograft cytotoxicity in vivo in that it begins at five days and peaks at seven to eight days after grafting. This time sequence is also indicated in the deterioration of the vascular endothelium of the graft (Liem and Jerusalem, 1970), which lends support to the nonspecificity of the reaction, since the vascular endothelium is derived from host tissue.

The cytotoxic activity is characterized by being independant of complement, independant of anti-immunoglobulin sera, and susceptible to sera prepared against thymocytes (Cerottini et al. 1971 and Cerottini et al. 1970). Such a substance, therefore, probably acts in a different manner than most antibodies.

Several attempts have been made to isolate and study this reactive "lymphotoxin". Kolb and Granger (1970) found a substance in mice which was heat and pH stable, acting like a protein with a molecular weight of 90-150,000. It did not require complement, and its heat stability indicated that it was not an immunoglobulin. Furthermore, these investigators stated that there were differing physical and chemical properties for such a substance in different species of animal. Kramer and Granger (1972) found that mouse macrophages could be induced in vitro to produce a cytotoxic

substance which cross-reacted with antibody specific for mouse lymphotoxin. An extensive investigation into the nature of mouse lymphotoxin by Williams and Granger (1969) indicated it to be a negatively charged protein of 90-100,000 molecular weight which acts progressively over 36-48 hours, is temperature and concentration dependant, does not bind strongly to target cells, and is nonimmunospecific in its action. The mechanism of action appears to be lysis of the cell membrane. If indeed such a substance is produced in the graft bed, it is obvious that this would contribute to the rejection process, and its nonspecificity would account for the destruction of host tissue (vascular endothelium) as well.

In addition to cell-mediated cytotoxicity, there is evidence that systemic humoral antibody is produced against a primary allograft in vivo. Canty and Wunderlich (1971) analyzed mouse serum daily following skin or tumor grafting and noted a rise in titer from 0 to 1/160 in seven days. This "serum cytotoxicity" was detected by release of Cr^{51} from target cells incubated with the sera. Additional evidence for a humoral allo-antibody is given by experiments in which spleen cells are cultured in the presence of allo-antigen, producing plague-forming cells (Fuji et al. 1971) which can inhibit allogeneic tumors in the presence of rabbit complement (Cerottini et al. 1971). Hamilton et al. (1971) believe that the humoral mechanism is insufficient to reject

an allograft by itself. Nevertheless, mouse skin allografts on alymphatic pedicels, although they survive longer, eventually shrivel and die just as surely as do allografts in contact with sensitized lymphocytes (Tilney and Gowans, 1971).

It appears then, that the basophilic cytoplasm of the hypertrophied lymphocytes of the graft bed (confirmed by electron microscopy to contain numerous free ribosomes [Walker et al. 1964]) may be producing a substance beneath the graft which acts as does lymphotoxin in vitro, and that this is a major factor in allograft rejection. Demonstration of protein production by the lymphocytes of the graft bed would support this hypothesis.

That radioactive labeling techniques using tritiated leucine can demonstrate protein production has been established by numerous investigators as exemplified by Reid and Heald (1970). These investigators, performing fractionation studies, noted that tritiated leucine was incorporated only into the protein fraction of the tissue and not into the carbohydrates or fats. Melchers (1970) noted that H^3L was not incorporated into the carbohydrate fractions of immunoglobulin molecules, but did appear in myeloma proteins of plasma cell tumors. Radioautography with tritiated leucine has been used to demonstrate protein production in lymph node cells in response to antigen (Slonecker, 1969).

No research has been reported which demonstrates protein production by hypertrophied lymphocytes in an in vivo graft rejection system.

MATERIALS AND METHODS

I. Animals

Animals used in this experiment were inbred A/J (H-2a) and C3H (H-2k) adult female mice*, which were between 12 and 24 weeks of age and weighed 20-28 gm at the time of skin grafting. These mice were maintained in individual cages following grafting and were housed in air conditioned quarters and given food and water ad libitum. Ether was used for anesthesia in all operative procedures.

II. Skin grafting

Allografts consisted of skin transplanted between A/J and C3H mice. Autograft controls consisted of skin grafts transplanted from one location to another on the same mouse.

Hair was clipped from the dorsal thorax of anesthetized animals and areas larger than the anticipated graft were shaved and cleansed with 70% alcohol. Surgical steps were carried out alternately on donor and host in order to minimize the time between removal of the graft from the donor and placement on the host. Cleaned instruments were soaked in 70% alcohol before operating and care was taken not to touch the graft or graft bed except with cleaned instruments

*Jackson Memorial Laboratories, Bar Harbor, Maine.

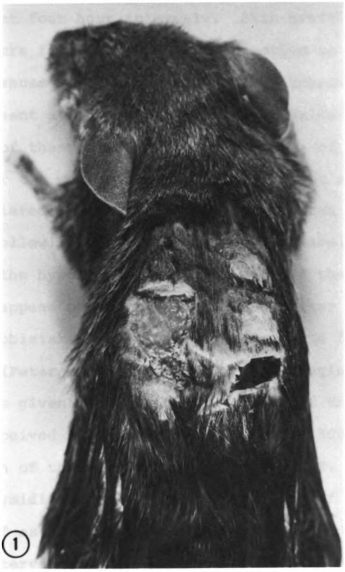
during the grafting procedure. The skin in a 1 cm^2 area was held gently with a forceps and a cut made on three borders of the graft with a small scissors to the depth of the panniculus adiposus. One corner of the resulting flap was lifted slowly with the forceps while the panniculus adiposus was split beneath the graft with a sharp scalpel. Thus the panniculus carnosus with its relatively rich vascularity was left intact in the graft bed. When the flap was completely separated, the fourth border was cut and the graft removed and immediately placed on the host whose graft bed had been prepared in the same way. The graft and surrounding skin were then covered with a layer of Flexible Collodion*, which was allowed to dry before the animal was returned to its cage. This method of split thickness grafting was the same as that recommended by Billingham (1961), except for the Collodion dressing.

A total of 10 mice was used in this experiment and each was given either six or eight skin grafts (three allografts and three autografts, or four allografts and four autografts) as illustrated in Figure 1. Allografts were placed on the animal's right side and autografts were placed on its left. This was done in order to conserve isotope and animals, and also to provide an internal control so that an allograft and autograft biopsided at a given time would be from the same animal. It also provided a control for the

*J.T. Baker Chemical Company, Phillipsberg, New Jersey.

possibility that cells of different animals might react in a slightly different time sequence. With several grafts on a single animal, the timing between biopsies would yield a more acurate estimate of cell and protein turnovers than if a single animal were sacrificed at each given time.

Figure 1. C3H mouse which had been given three allografts and three autografts. Two autografts (on the left) have been removed for histologic analysis, as has one of the allografts. Two allografts remain in place on the upper right, and one autograft on the upper left.



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III. Radioisotope injections

Four animals serving as the main experimental group were injected with both tritiated thymidine* and tritiated leucine**. The thymidine was given subcutaneously in three equal doses at four hour intervals. Skin grafting was performed 24 hours following the last injection so that hematopoietic tissues would be labeled and no unbound label would be present at the time of grafting (Walker and Goldman, 1963). Two of these mice were given a total of 300 µc each and the other two were given a total of 600 µc each of H^3T.

Tritiated leucine injections were given on the fourth day following grafting, in order to label the protein produced by the hypertrophied lymphocytes of the graft bed, which first appear on day three or four (Walker and Goldman, 1963 and Jakobisiak, 1971), and whose products first appear on day five (Peter and Feldman, 1972). A single dose of 200 µc of H^3L was given subcutaneously to each of the two mice which had received 300 µc H^3T, and a dose of 500 µc H^3L was given to each of those receiving 600 µc of H^3T.

A thymidine control group consisting of three animals was given H^3T as above in three subcutaneous injections at four hour intervals 24 hours before grafting. Two of these mice were given a total of 300 µc of H^3T each, and one was

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*Thymidine-methyl-H^3, specific activity 2.0 curies/mM, New England Nuclear, Boston, Massachusetts.
**L-leucine-4, 5-H^3(N), specific activity 5 curies/mM, New England Nuclear, Boston, Massachusetts.

given a total of 600 μc of H^3T. No leucine was given to the mice of this group.

A leucine control group consisting of three animals was not given thymidine before grafting, but did receive tritiated leucine on the fourth day after grafting, as had the experimental group. One of these mice was given 200 μc of H^3L and two were given 500 μc of H^3L each. Table 1 summarizes the radioisotope doses in all animals.

Table 1. Summary of radioisotopes injected.

	Animal #	Strain	H^3T	H^3L
Experimental group	1	C3H	300 μc	200 μc
	2	A/J	300 μc	200 μc
	3	C3H	600 μc	500 μc
	4	A/J	600 μc	500 μc
Thymidine control group	5	A/J	300 μc	
	6	C3H	300 μc	
	7	A/J	600 μc	
Leucine control group	8	C3H		200 μc
	9	A/J		500 μc
	10	C3H		500 μc

IV. Graft biopsy

In the experimental group and the leucine control group, at least one allograft and autograft pair were removed at each of the following times: one hour and two hours after H^3L injection on day four after grafting, and days five, six, eight, and ten after grafting. In the thymidine control group at least one allograft and autograft pair were removed at each of the following times: days three, four, five, six, eight, and ten after grafting. Table 2 contains a complete biopsy schedule for all animals.

Table 2. Graft biopsy schedule. An X indicates one allograft and one autograft were removed at this time.

Animal #	Strain	Days after grafting					
		3	4	5	6	8	10
1	C3H		X*	X	X		
2	A/J				X	X	X
3	C3H		X**	X	X		
4	A/J		X*			X	X
5	A/J		X	X	X		X
6	C3H	X					
7	A/J		X	X	X	X	X
8	C3H		X*	X	X		X
9	A/J		X*			X	X
10	C3H		X**	X	X		

*One hour after H^3L injection.
**Two hours after H^3L injection.

At each time interval, the animal was anesthetized with ether and the graft area cleaned gently with 70% alcohol. The same procedure for instrument cleaning and graft handling were used for biopsy as had been used in grafting (above). Care was taken not to disturb adjacent grafts or remove their Collodion coating. The skin in the area adjacent to a graft was lifted with a forceps and incised with a small scissors. A cut was made through the skin to the depth of the skeletal musculature. The incision was made in the non-grafted host skin completely surrounding the graft so that the biopsy included normal skin on all sides of the graft. The section was then lifted with the forceps and attachments to the skeletal musculature were severed with a scalpel in an attempt to include all available tissue with the biopsy. The tissue was then placed in 10% buffered formalin for fixation. Topical antibiotic powder* was applied to the resulting wound and covered with gauze which was then held in place by a layer of Flexible Collodion. Blood smears were taken at the time of each biopsy in order to correlate the radioautographic phenomena of the peripheral circulation with that of the graft bed biopsied. Samples were taken by stabbing the cleansed tail vein with the point of a #11 scalpel blade.

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*Neosporin brand of Polymyxin B, Bacitracin, and Neomycin, Burroughs Wellcome and Company, Incorporated, Tuckahoe, New York.

V. Histologic techniques

A. Skin grafts

After fixation in 10% buffered formalin for 24 hours, the tissues were dehydrated in ethanol, infiltrated, and embedded in a glycol methacrylate mixture*. This method was chosen because of the necessity of making two micra sections so that the resolution under light microscopy would be sufficient to allow accurate distinction between cytoplasm and nuclei of the cells involved, since thymidine would label nuclei and leucine presumably would label cytoplasm, where most protein production would occur. Once polymerization was complete, the tissues were sectioned at two micra with a Sorvall JB-4 microtome**. Sections were floated in double distilled water on cleaned slides and dried on a hot plate at 50°C. Slides were then processed for radioautography (see C below). Following developing, slides were stained in hematoxylin and eosin and cover slipped with either Histoclad*** or Permount****.

B. Blood Smears

Blood smears were made on cleaned slides and allowed to air dry. Slides were then dipped in a 0.05%

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*JB-4 Plastic Embedding Kit, Ivan Sorvall, Incorporated, Newton, Connecticut.
**Ivan Sorvall, Incorporated, Newton, Connecticut.
***Clay-Adams Incorporated, New York, New York.
****Fisher Scientific Company, Fair Lawn, New Jersey.

solution of Parlodion* in absolute ethanol in order to insure adherence to the slide. Thus prepared, the slides were coated for radioautography (see C below). After developing, the slides were stained by flooding for 20 minutes with Giemsa stain diluted with phosphate buffer 1/20. Slides were then rinsed with distilled water, allowed to dry, dipped in xylene, and cover slipped with Histoclad.

C. Radioautography

Prepared slides were coated in a dark room with Kodak type NTB_2 nuclear track emulsion**. The procedure for coating, drying, storage, and developing of slides was that detailed by Walker (1959) except that the celloidin coating was omitted. Slides were exposed for varying intervals from one to eight weeks, depending on radioisotope dosage.

VI. Statistical methods

All grain counts were performed on slides which had been exposed for eight weeks in the case of mice given 300 μc of H^3T and/or 200 μc of H^3L, and four week exposures were used for those given 600 μc H^3T and/or 500 μc of H^3L. Counts were performed on cells found in randomly selected 2500 μ^2 areas of the graft bed.

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*Mallinckrodt Chemical Works, St. Louis, Missouri.
**Eastman Kodak Company, Rochester, New York.

The thymidine label was evaluated by counting silver grains located over cell nuclei and measuring the nuclear area, thereby determining the nuclear grain density. Nuclear area was determined by estimating the nuclear area to the nearest 5 μ^2 in comparison to an ocular grid on the microscope which was composed of 100 μ^2 squares. Reutilization of H^3T label by proliferating fibroblasts was accounted for by comparing the nuclear grain density with that seen in endothelial cell nuclei (the reutilization index). Graft bed cells with a nuclear density greater than the reutilization index plus twice its standard deviation were considered to be hypertrophied lymphocytes.

The leucine label was evaluated by measuring cytoplasmic area and counting silver grains (cytoplasmic grain density) for cytoplasm directly associated with a radioactive nucleus. Cytoplasmic area was determined by comparison to an ocular grid and estimated to the nearest 5 μ^2. Comparisons of cytoplasmic counts and densities for a given animal over a given period of time were analyzed according to mean and standard deviation, using $p < 0.05$ for statistical significance. Formulae used to calculate these data are given in the appendices.

RESULTS

I. Graft survival

All animals used in this experiment remained healthy throughout the manipulations and suffered no obvious adverse systemic effects from the radiation used nor from the multiple grafting and biopsying. The clear Collodion coating over the grafts allowed observations of the grafts and any changes occurring without disturbing the grafted tissue. This coating was also effective in holding the grafts in place until biopsy. Even allografts, which would likely have been sloughed sooner, were maintained up to ten days after grafting. All grafts were observed to "take" initially; indeed at three to four days after grafting, the only differences seen between allografts and autografts were the variations in pigmentation between C3H and A/J skin. As time intervals increased between four and ten days after grafting, allografts were observed to become more darkly colored and dried, as well as shrunken in size, whereas autografts maintained the healthy appearance noted at three to four days after grafting.

II. Histology

Histologic observations were aided greatly by the JB-4 plastic embedding technique. Sections cut at two micra permitted a great degree of resolution, especially at high

power and under oil immersion. Whereas in seven micra paraffin sections the plane of focus must be raised and lowered in order to view all cells of the section, the two micra sections showed all the details of all the cells present, without readjusting the focus. It was still necessary, however, to raise the plane of focus in order to view the silver grains in the layer of emulsion. Nevertheless, the greater resolution was also beneficial in this situation, because it could easily be determined whether a given silver grain was originating from the nucleus or cytoplasm of the cell beneath. In contrast, using paraffin sections, one would have to consider the possibility that a layer of cells deep to the surface layer was producing the radioactivity observed.

Microscopic observations of the grafts and graft beds revealed several notable features. Allografts and autografts are described separately and appropriate comparisons drawn.

A. Allografts

At three to four days after grafting, the grafted tissue was still easily discernable from the host tissue (compare Figures 2 and 3). The graft itself now had only a thin layer of epithelium, many of the cells of which appeared to be necrotic, but some still appeared essentially normal. The epithelia of allografts and normal skin are illustrated in Figures 7 and 8. Several neutrophils were seen to have invaded the graft and were especially numerus in areas adjacent to host skin at the edges

of the graft. Typically, a densely packed area of neutrophils was situated within the junction of host and grafted skin (see Figure 3). The layers of dermis and panniculus adiposus were evident within the grafted skin, but as the base of the graft was approached, an increasing proportion of cells was encountered with basophilic cytoplasm and medium to large basophilic nuclei. Cells of this type also comprised a dense layer of three to four cells in depth between the graft panniculus adiposus and the host panniculus carnosus. Individual cells had a variety of nuclear shapes and sizes, but many nuclei were noted averaging about 30 μ^2 in area and containing numerous small nucleoli. These cells appeared to be flattened, with their broad surface parallel to the surface of the graft (Figures 12 and 16). Several larger basophilic cells were also noted here. The panniculus carnosus contained numerous muscle fibers which were smaller in diameter than those of adjacent uninjured skin, and which occasionally had centrally placed nuclei (Figures 13 and 14). Below the panniculus carnosus was a layer of widely spaced basophilic cells, this layer being somewhat thicker in the area immediately beneath the graft than beneath uninjured skin.

By eight to ten days, the graft, although remaining in place, was completely necrotic with no evidence of living cells present (see Figure 4). The dense layer

Figure 2. Low power photomicrograph of a graft immediately after placement. The intact skin is present on the right, and the grafted skin on the left. Note that the panniculus carnosus of the host remains intact in the graft bed. .3mm

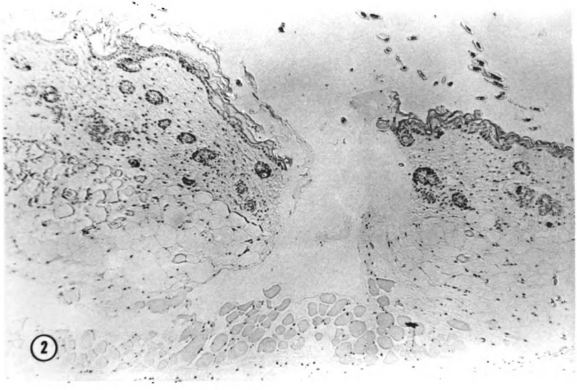
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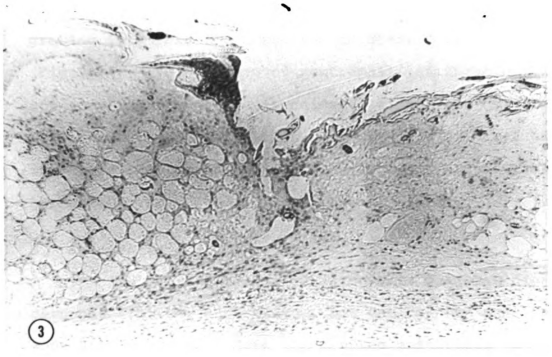
Figure 3. Allograft three days after placement. The graft is on the right and has a thin, necrotic epithelium. The dark area at the edge of the normal epithelium is a clump of neutrophils, typically seen filling the junction between graft and host skin. .3mm

Figure 4. Allograft ten days after placement. The graft (above) is necrotic and contains many neutrophils (black area). The host epithelium has grown in from the left (arrows) and separates the graft from the host. .3mm



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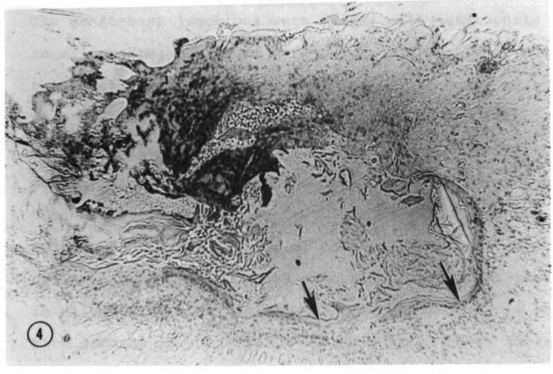

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of basophilic cells was still present between graft and host tissue, and, in addition, host epithelium was seen growing into this area from the adjacent host skin (Figure 4). The pattern of neutrophil infiltration did not seem consistant in all animals, since some allografts had increasing concentrations with increasing time, and in others the concentration of neutrophils did not change appreciably from the three or four day level.

B. Autografts

The grafted tissue of autografts was also discernable from the host tissue, but not for the same reasons as allografts. Here the grafted epithelium remained healthy looking without evidence of necrosis (Figure 5). The graft-host junctions were filled with neutrophils as in allografts, but there were fewer neutrophils invading the graft itself. The layer of basophilic cells was present between the graft panniculus adiposus and host panniculus carnosus (Figure 10), but did not appear as densely packed as that beneath allografts. Consequently the basophilia seemed less prominent in this area for autografts than it did for allografts. The individual cells in this area appeared generally larger than those in allografts, with nuclei averaging about 50 μ^2 in area, with some as large as 100 μ^2 (Figure 11). These nuclei contained one or two large nucleoli, compared to the more numerous and smaller nucleoli noted in cells beneath

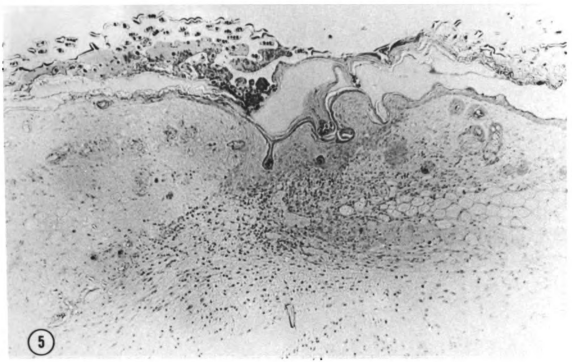
allografts (see Figures 15 and 16). The host panniculus carnosus was similar to that noted beneath allografts in that it had numerous small fibers, some of which contained centrally placed nuclei. The loose fascial layer beneath the panniculus carnosus looked the same as that observed for allografts. Autografts biopsied at later time intervals did not have the changes noted for allografts. Here the grafts continued to look healthy, and the distinctions between graft and host tissue gradually diminished. At day ten after grafting, the only difference seen was the elevation of the grafted tissue above the level of the normal skin (Figure 6).

III. Radioautography

As detailed above, some of the animals in each experimental group were given at least twice as much radioactive isotope as the others, and consequently could be observed after a shorter exposure time. In addition to this exposure and dosage difference, tissues from those mice given the lower dosages (300 μc tritiated thymidine and/or 200 μc tritiated leucine) were processed before the JB-4 embedding technique was perfected. As a result, many of the sections were too small or too poorly oriented to provide the volume of data needed to yield statistical significance. Nevertheless, these preliminary data suggested all the main points described below, and the subsequent statistical analyses,

Figure 5. Autograft three days after placement. The graft (on the left) has a thinner epithelium than normal skin, but most of its cells are normal.

Figure 6. Autograft ten days after placement. The epithelium of the graft (right) appears similar to normal epithelium (Figure 8). Numerous basophilic cells are present in the graft bed, but are less dense than those in allografts (compare Figures 9 and 10).



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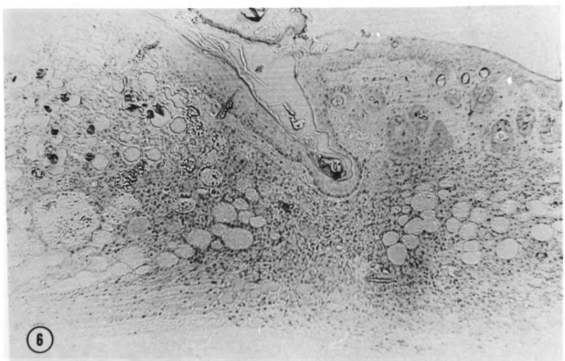

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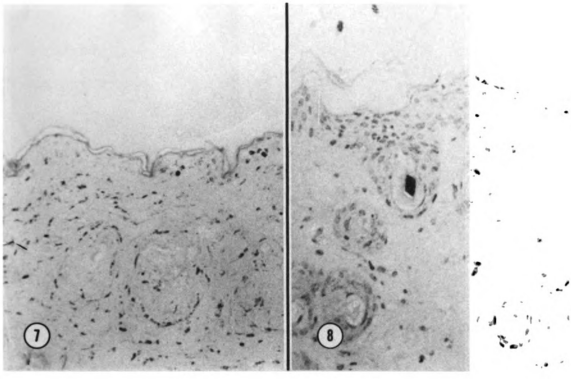
Figure 7. Thin epithelium of a three day allograft. Most of the cells are already necrotic, as are the hair follicles (compare with Figure 8). [.1mm]

Figure 8. Normal epithelium from the surrounding area skin. Here, all cells appear normal. Late autografts eventually regained this appearance (see Figure 6). [.1mm]

Figure 9. Basophilic cell layer in a six day allograft bed. Cells appear smaller and more densely packed than in a comparable autograft bed (Figure 10). [50 μ]

Figure 10. Basophilic layer in a six day autograft bed. Cells appear relatively large and are not as densely packed as in allografts. [50 μ]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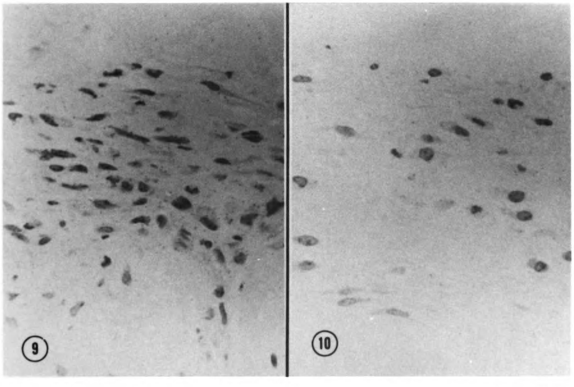
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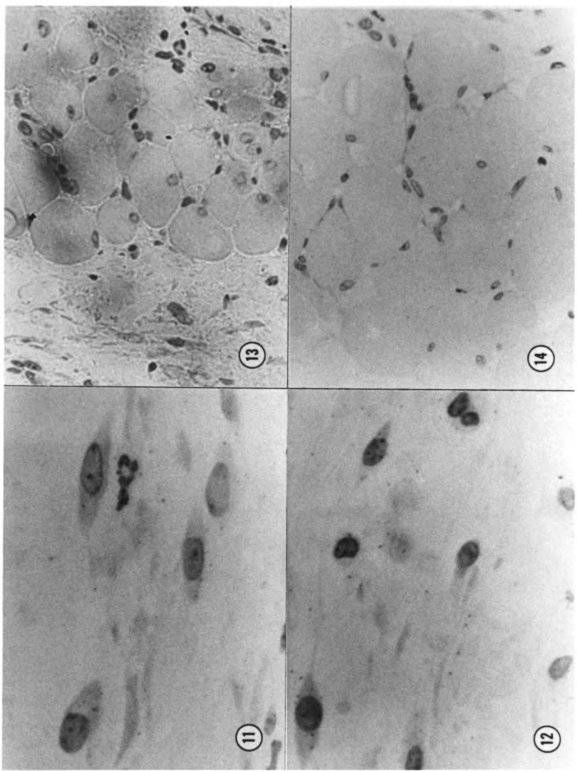
Figure 11. High power photomicrograph of basophilic cells in the graft bed of a six day autograft. Note the prominent nucleoli in most cells. ⌊ 25 μ ⌋

Figure 12. Basophilic cells in a six day allograft bed. Cells are small with no prominent nucleoli. Note the parallel orientation of the elongated cytoplasm. ⌊ 25 μ ⌋

Figure 13. Panniculus carnosus beneath the graft area. Fibers are smaller than normal (Figure 14). Note central nuclei in several fibers at the bottom of the photograph. ⌊ 50 μ ⌋

Figure 14. Panniculus carnosus beneath uninjured skin. The fibers are of normal size and have the normal nuclear configuration. ⌊ 50 μ ⌋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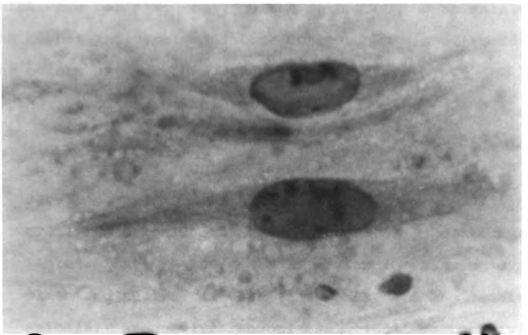
11
12

Figure 15. Oil immersion photomicrograph of large basophilic cells beneath a six day autograft. ⌊ 10 μ ⌋

Figure 16. Basophilic cells beneath a six day allograft. Cells appear more flattened, with more dense nuclear material. Note parallel cell orientation which is also parallel to the graft (not visible).

⌊ 10 μ ⌋



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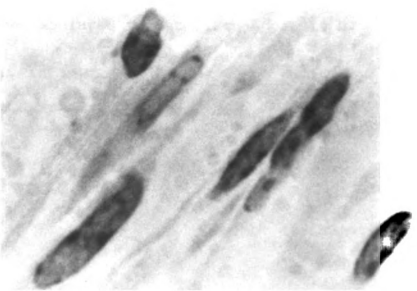


(16)

which were performed only on the data from the animals with the higher radioactive isotope dosages, were used to support the points as described.

A. Leucine labeling (Figures 17 and 18)

In the leucine control group (those injected with only tritiated leucine) a rather consistant pattern of labeling was noted in that most of the silver grains were overlying cytoplasmic areas, rather than nuclear areas. Table 3 lists the percentages of label overlying cytoplasm and nuclei for each graft on two of the leucine animals. Areas were randomly selected from the living tissue of the graft bed or surrounding normal dermis. There was no consistant change in this distribution pattern with increasing time after injection, nor was there any difference between allografts and autografts. Counts are tabulated in detail in Appendix D.

Table 3. Distribution of tritiated leucine label between cytoplasm and nuclei. Each count is from a randomly selected area of graft bed or adjacent normal dermis. Parentheses indicate grain densities (grains/μ^2).

animal number	allo-graft	auto-graft	days after grafting*	% label over cytoplasm	% label over nuclei
9	X		4**	80 (.25)	20 (.14)
9	X		8	83 (.19)	17 (.11)
9	X		10	80 (.14)	20 (.12)
9		X	4**	85 (.19)	15 (.18)
9		X	8	82 (.19)	18 (.15)
9		X	10	82 (.17)	18 (.16)
10	X		4***	86	14
10	X		5	80	20
10	X		6	78	22
10		X	4***	78	22
10		X	5	78	22
10		X	6	86	14

*Animals were injected with H^3L on day 4 after grafting.
**One hour after H^3L injection.
***Two hours after H^3L injection.

In order to obtain a more accurate estimate of the relative proportions of leucine in cytoplasm and nuclei, the areas of these cell subdivisions must be considered, thus actually comparing cytoplasmic radioactive density with nuclear radioactive density rather than absolute grain counts. This was performed for animal 9 and the values appearing in parentheses in Table 3 indicate only a slightly higher concentration of label in cytoplasm than in nuclei.

B. Thymidine labeling (Figures 19 and 20)

In the animals which were given only tritiated thymidine, a direct contrast to the leucine labeling pattern was noted. Here, the label was concentrated almost exclusively over nuclei, and was light over cytoplasm. Table 4 lists the percentages of silver grains overlying cytoplasm and nuclei for each graft on animal 7. This distribution was also consistant over time and between allografts and autografts (data given in Appendix D).

Table 4. Distribution of tritiated thymidine label between cytoplasm and nuclei in animal number 7. Random areas of the graft beds or adjacent normal skin.

allograft	autograft	days after grafting*	% label over cytoplasm	% label over nuclei
X		4	23	77
X		6	24	76
X		8	19	81
X		10	21	79
	X	4	21	79
	X	6	24	76
	X	8	18	82
	X	10	21	79

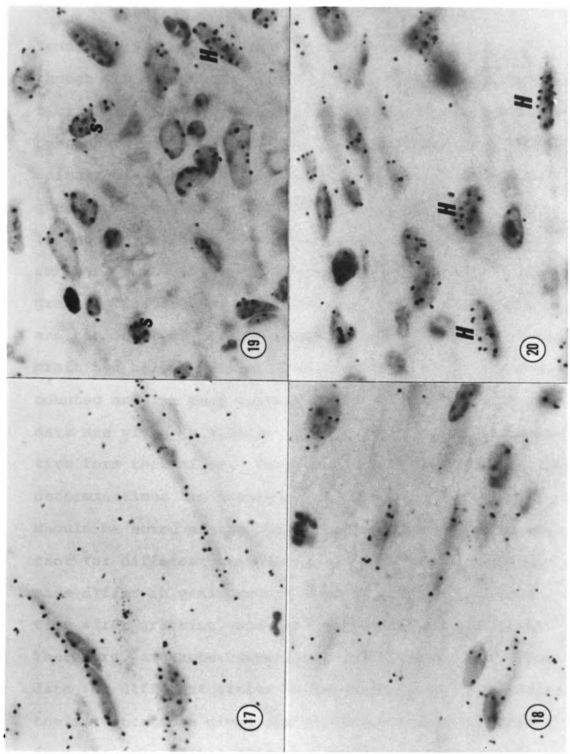
*Injected with H^3T one day before grafting.

Figure 17. Basophilic allograft bed cells from animal 10 (leucine group) one day after injection of H^3L. Most of the label is visible overlying cytoplasm. ⌊ 20μ ⌋

Figure 18. Basophilic cells from five day autograft on animal 10 (leucine group). As in allografts, the label concentrates over the cytoplasm. ⌊ 20μ ⌋

Figure 19. Basophilic cell layer of a four day allograft bed from animal 7 (thymidine group). H - hypertrophied lymphocyte. s - smaller lymphocytes. ⌊ 20μ ⌋

Figure 20. Basophilic cell layer of a 10 day allograft bed from animal 7 (thymidine group). Three hypertrophied lymphocytes (H) are easily visible and show heavy labeling. Note that in both of these figures almost all of the silver grains overlie nuclei. ⌊ 20μ ⌋


S
S
H
17
18
19
H
H
H
20

C. Labeling within the graft itself

Preliminary data indicated that allografts tended to accumulate label with increasing time, even though the tissue was becoming increasingly more necrotic; whereas autografted tissue maintained a relatively constant label. This did not seem to be as true for animals given only H^3T as for those given H^3L. Subsequent data confirmed these findings. Ten unit areas (areas of the microscope grid under oil immersion measuring 2500 μ^2 each) were selected at random from the grafted tissue located between the graft epidermis above and the layer of densely packed basophilic cells of the graft bed below. Silver grains in these unit areas were counted and the mean calculated for each graft. These data are given in tabular form in Table 5 and in narrative form thereafter. Comprehensive data and statistical determinations are tabulated in detail in Appendix A. It should be noted at the outset that data are not consistant for different animals, i.e. grafts from different mice differ in grain counts even though type of graft, time after grafting, and isotope dosage are identical. Therefore, accurate comparisons can be drawn only from data for different grafts on the same mouse. Therefore the illustrations given are photomicrographs of grafts from the same animal (Figures 22-27).

Table 5. Radioactivity within grafted tissue. Each mean represents the number of silver grains in an average 2500 μ^2 area of graft tissue.

animal number	radioisotope	days after grafting	mean allograft radioactivity	mean autograft radioactivity	mean dermis (normal) radioactivity	mean fog
9	H^3L	4*	18.6	***	57.3	8.4
9	H^3L	8	38.9	44.6	44.2	8.9
9	H^3L	10	43.2	37.8	36.2	7.4
10	H^3L	4**	14.5	31.8	62.2	8.6
10	H^3L	5	31.1	37.6	58.5	8.8
10	H^3L	6	57.6	36.2	54.8	9.5
4	H^3T and H^3L	4*	38.8	52.8	57.3	9.8
4	H^3T and H^3L	8	***	38.4	39.9	9.6
4	H^3T and H^3L	10	47.6	32.5	32.8	8.9
3	H^3T and H^3L	4**	42.0	55.1	60.9	8.6
3	H^3T and H^3L	5	50.5	35.7	42.6	7.8
3	H^3T and H^3L	6	66.7	34.7	37.4	8.5
7	H^3T	4	16.0 (5.2)****	15.8 (5.0)	18.0 (7.2)	10.8
7	H^3T	6	13.4 (4.1)	11.8 (2.5)	13.7 (4.4)	9.3
7	H^3T	8	18.8 (10.6)	11.4 (3.2)	10.9 (2.7)	8.2
7	H^3T	10	12.0 (3.3)	11.4 (2.7)	10.0 (1.3)	8.7

*One hour following H^3L injection
**Two hours following H^3L injections
***Insufficient tissue available
****Figures in parentheses are after fog substraction

In animal 9, a comparison of allografts and autografts indicates that allograft tissue gradually increased in radioactivity, especially in the initial four day interval, the same time that autografts and normal skin dermis were decreasing their radioactivity. In animal 10 (given only H^3L) the same pattern was noted, even though biopsy intervals were only 24 hours. The increase in autograft radioactivity seen between four and five days is the only instance where an autograft was observed to increase in grain density, and possible reasons will be discussed below.

Animals 3 and 4 were given both tritiated thymidine and leucine; however, a comparison of animal 3 with animal 10 and animal 4 with animal 9 (because these were biopsied at parallel time intervals) reveals that the addition of H^3T did not consistantly increase the grain counts in the grafts, nor in the normal dermis. Radioactivity in allografts was again noted to increase (Figures 22 and 23) and in autografts and normal skin to decrease with increasing time after grafting and injection (Figures 24-27). In these two animals (3 and 4), the grain counts over autografts and normal dermis were essentually parallel in their declines, as can be seen in Table 5.

Background fog remained relatively constant for all time intervals and between different animals. The

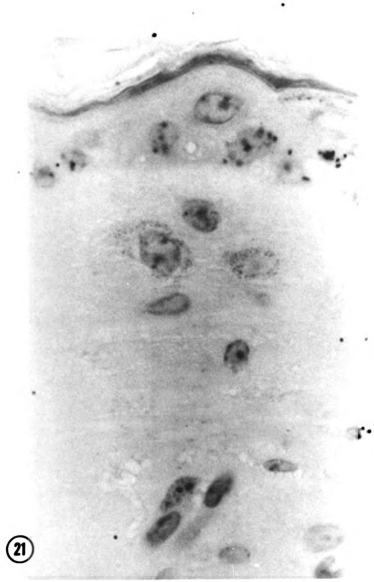
graft radioactivity in animals given H^3L was elevated to such an extent that these consistantly low fog levels did not present an important factor in evaluating changes in the graft radioactivity. However, in the case of animal 7 where only H^3T was given, the graft radioactivity was only slightly elevated above background fog levels, and when these were subtracted, no consistant pattern of decrease or increase over time was seen. These data indicate that thymidine contributed an insignificant amount of radioactivity to the graft tissue (compare Figure 21 with Figures 22-27).

Statistical significance ($p < .05$) was obtained for each of the increases seen in allografts except for that between day 8 and 10 in animal 9. Each of the decreases seen in autograft radioactivity was also statistically significant except for those between day 5 and 6 in animal 10, and day 5 and 6 in animal 3. These calculations are detailed in Appendix A.

Two generalized statements can be made to summarize the above data. In animals given H^3L four days after grafting: (1) allograft radioactivity increased with time, while autograft and normal dermis radioactivity decreased; and (2) allografts were initially less radioactive than either autografts or normal dermis, but at late intervals allografts were more radioactive than either autografts or normal dermis (see Figures 21-27).

Figure 21. Allograft tissue from animal 7 (H^3T), six days after grafting. Epithelial cells are still intact and have incorporated some H^3T. There is very little radioactivity below the epithelium.

20μ



(21)

Figure 22. Allograft tissue from animal 4 (H^3T and H^3L), four days after grafting and one hour after the H3L was injected. Epithelium is beginning to become necrotic. The graft has very little radioactivity at this early stage. 20μ

Figure 23. Allograft tissue from animal 4. ten days after grafting. The thin line of keratin (k) at the top of the photograph is all that remains of the epithelium. The tissue beneath the epithelium now contains the remains of necrotic cells plus a great increase in radioactivity.

20μ

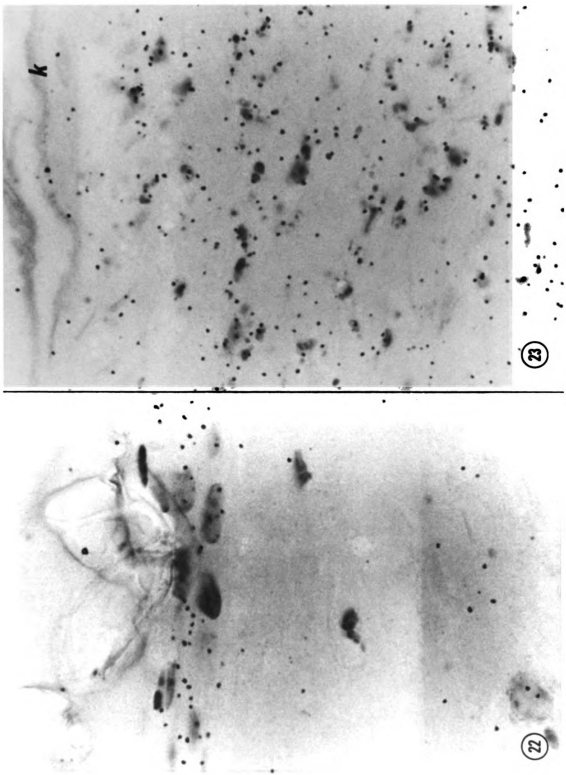

k
23
22

Figure 24. Autograft tissue from animal 4, four days after grafting and one hour following the H^3L injection. The radioactivity is greater in both epithelium and dermis than that noted for the comparable allograft (Figure 22). 20μ

Figure 25. Autograft tissue from animal 4, ten days after grafting. Radioactivity has decreased slightly from the four day level (Figure 24). 20μ

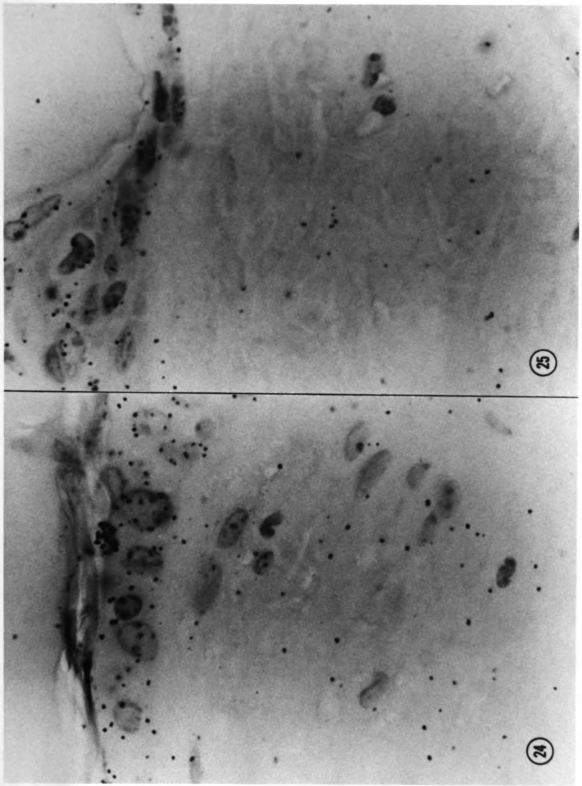

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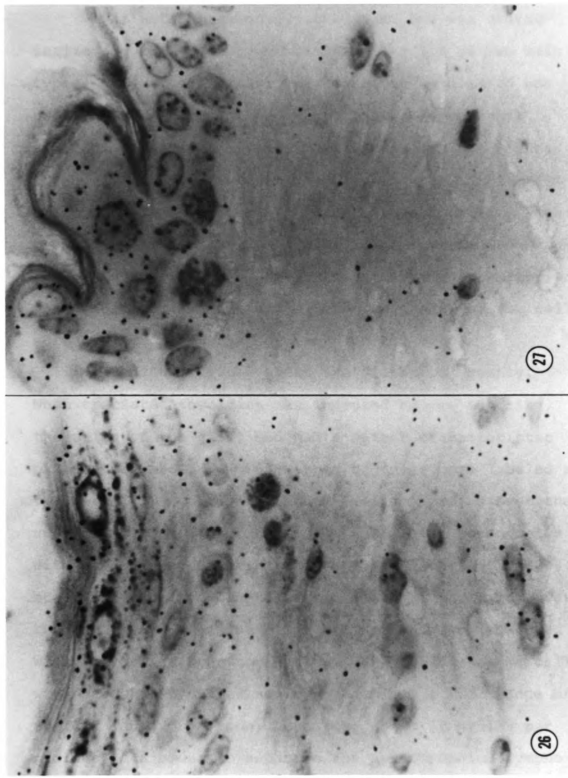
Figure 26. Normal skin from animal 4, eight days after grafting. All cells are alive, and in the epithelium the radioactive materials have been incorporated into the keratin layer. The cells of the graft dermis show their normal protein production. 20μ

Figure 27. Normal skin from animal 4, ten days after grafting. This tissue shows a decrease in radioactivity of both epithelium and dermis, as would be expected due to the normal protein turnover rate. 20μ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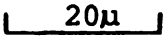
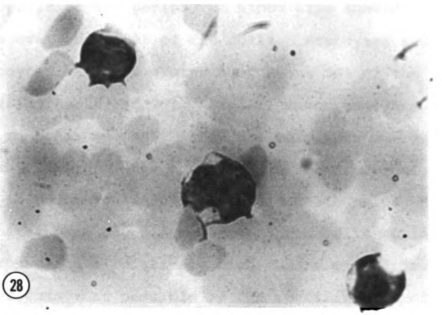
D. Labeling within the graft bed

As noted previously, the graft bed was characterized by the presence of basophilic cells of two main types; those whose nuclei were large and contained one or two large nucleoli; and those which had somewhat smaller nuclei with several small nucleoli (see Figures 15 and 16). Both of these cell types were seen in allograft and autograft beds, but there seemed to be a higher proportion of the smaller nuclei in allografts and larger nuclei in autografts. Still, these histologic criteria were insufficient to clearly determine which of the cells were hypertrophied lymphocytes from the blood.

Radioautography aided the situation greatly. When tritiated thymidine was injected before grafting, the cells of the graft bed had a rather characteristic pattern of labeling. Four types of cells were labeled as follows: (1) neutrophils had 5-6 silver grains over the nuclei of about 80% of the cells; (2) large round cells with kidney shaped nuclei and varying amounts of pale-staining cytoplasm generally had 9-10 silver grains over their nuclei; (3) endothelial cell nuclei were observed with 2-4 grains over the nucleus; and (4) the large cells with basophilic nuclei and cytoplasm had a wide range of nuclear label from none, to a few; up to 12 grains per nucleus. In order to separate the hypertrophied lymphocytes from the fibroblasts in this last population, comparisons were drawn to the labeling seen in smears of

Figures 28 and 29. Blood smears from animal 7 (H^3T), four days after grafting. Note that the large monocyte (with kidney shaped nucleus) in Figure 28 and the large round cell in Figure 29 are labeled, while the small lymphocytes in Figure 28 are not labeled. The neutrophil in Figure 29 has silver grains over it which are not visible in the photograph because of its dense staining.





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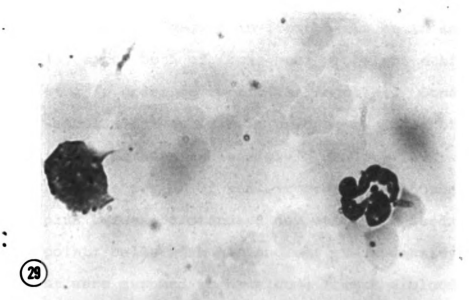

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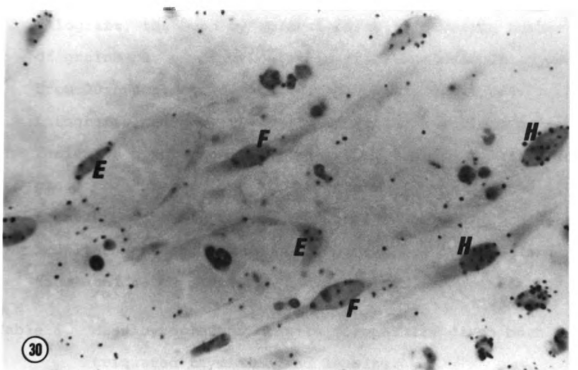
peripheral blood. Three days after H^3T injections, the neutrophils of the peripheral blood also showed 5-6 grains over about 80% of the cells. Monocytes and large lymphocytes had 8-10 grains over about 50% of the cells seen (Figures 28 and 29). Small lymphocytes were seen in great numbers, but only a few had more than one or two silver grains over the nucleus. In the graft bed, therefore, in order to identify the basophilic cells which had originated from blood lymphocytes, the cells would have to demonstrate a relatively high amount of radioactivity, greater than that possible by the reutilization of the degenerating neutrophil label. Cells with this heavy label would then correspond to the large lymphocytes of the peripheral blood in their labeling characteristics. It was assumed that reutilization of neutrophil label would be to about the same degree in fibroblasts as in endothelial cells, since both of these must proliferate after the grafting in order to repair the injuries. Consequently, endothelial cells were used as an index of reutilization. The mean nuclear grain density (average number of grains per μ^2 of nuclear area) of endothelial nuclei, plus twice its standard deviation was used as the cut off point; cells with the nuclear grain density above this point were assumed to have come from the blood. This procedure was carried out for each graft bed for all animals receiving tritiated thymidine, and the comprehensive data are tabulated in Appendix B. Figure 30 illustrates these types of cells.

Figure 30. Allograft bed cells from animal 3 (H^3T and H^3L), five days after grafting. Note that the two endothelial nuclei present (E) have silver grains over their nuclei, evidence of thymidine reutilization. Using these cells as a reference, it appears that fibroblasts (F) have reutilized thymidine to about the same degree, whereas hypertrophied lymphocytes (H) maintain a higher concentration of nuclear label. The cytoplasmic labeling is light in all of the cells shown here.

20μ


E
F
H
E
H
F
30

Once the hypertrophied lymphocytes were thus identifiable by their nuclear (thymidine) label, the animals also receiving tritiated leucine were analyzed in order to determine what happened to the protein in the cytoplasm of these cells in the graft beds. Cytoplasmic areas were measured for each cell (to the nearest 5 μ^2) and grain densities calculated. For each allograft, the mean cytoplasmic density (average number of grains per μ^2 of cytoplasmic area) was calculated from 20 hypertrophied lymphocytes of the graft bed. Autografts had very few cells meeting the nuclear criteria for hypertrophied lymphocytes, therefore mean cytoplasmic densities for each autograft were calculated from 20 basophilic cells regardless of origin or nuclear labeling pattern. The resulting data are outlined and tabulated below (Table 6) and in detail in Appendix C.

Table 6. Mean cytoplasmic density for animals given both tritiated thymidine and leucine.

animal number	days after grafting	hypertrophied lymphocyte cytoplasmic density (allograft)	autograft bed cytoplasmic density
4	4*	0.25	0.14
4	8	0.15	0.17
4	10	0.14	0.16
3	4**	0.19	0.15
3	5	0.15	0.15
3	6	0.13	0.15

*One hour after H^3L injection.
**Two hours after H^3L injections.

In animals given both tritiated thymidine and leucine (animals 3 and 4, Table 6), the hypertrophied lymphocytes of the allograft beds had a decreasing cytoplasmic density with increasing time. Each of the decreases in Table 6 was statistically significant ($p < .05$) except between day 8 and 10 in animal 4. Cytoplasmic densities for autografts on the same animals did not decrease, but remained relatively constant for the same time intervals. Typical allograft bed cells from these animals are illustrated in Figures 31 and 32. Typical autograft bed cells are illustrated in Figures 33 and 34.

These cytoplasmic densities reflect only the labeling due to H^3L and not to H^3T. In animals given only H^3thymidine there was no cytoplasm present which was labeled sufficiently to provide grain counts. Table 7 lists cytoplasmic densities for animals given only H^3leucine, and these values were similar to those seen where both isotopes were given (Table 6).

Table 7. Mean cytoplasmic densities for animals given only tritiated leucine.

animal number	days after grafting	allograft mean cytoplasmic density	autograft mean cytoplasmic density
9	4	0.25	0.19
9	8	0.19	0.19
9	10	0.14	0.17
10	4**	0.20	0.20
10	5	0.20	0.18
10	6	0.16	0.17

*One hour after H^3L injection.
**Two hours after H^3L injection.

Again the densities decreased more in allografts than autografts, but here there was no nuclear label and therefore a pure population of hypertrophied lymphocytes for allograft beds was not obtained, as it had been for values in Table 6.

The above data for cytoplasmic labeling can be summarized in two sentences. 1) In animals given H^3L, or H^3L and H^3T, cytoplasmic radioactivity declined more in allografts than in autografts. 2) In animals given both H^3L and H^3T, hypertrophied lymphocytes decreased in cytoplasmic density in allograft beds, whereas cytoplasmic radioactive density was relatively constant in autograft beds (see Figures 31-34). Background fog was not an important factor in these calculations because, averaging 10 grains per 2500 μ^2 area, it could contribute only .004 grains/μ^2 to a calculation of grain density.

Figure 31. Allograft bed cells from animal 3, four days after grafting. Two hypertrophied lymphocytes (H) are present with heavy nuclear labels and moderate labels over cytoplasm. ⌊ 20μ ⌋

Figure 32. Allograft bed cells from animal 3, six days after grafting. The hypertrophied lymphocytes (H) now have light cytoplasmic radioactivity. ⌊ 20μ ⌋


H
H
31

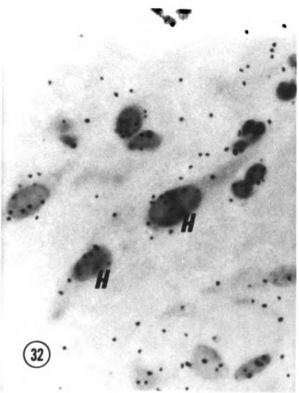
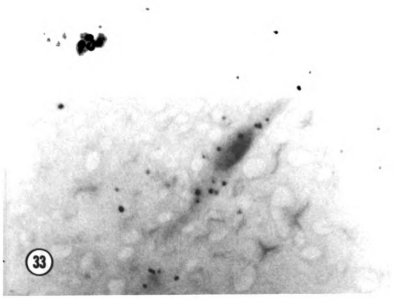

H
H
32

Figure 33. Autograft bed cell from animal 3, four days after grafting. The nucleus has light radioactivity (either H^3T or H^3L), and the cytoplasm is quite heavily labeled. [20μ]

Figure 34. Autograft bed cell from animal 3, six days after grafting. Both nucleus and cytoplasm are well labeled, but the nuclear density is not great enough to meet the criteria for a hypertrophied lymphocyte. The cytoplasmic grain density has decreased only slightly from that typical for the earlier intervals (Figure 33). [20μ]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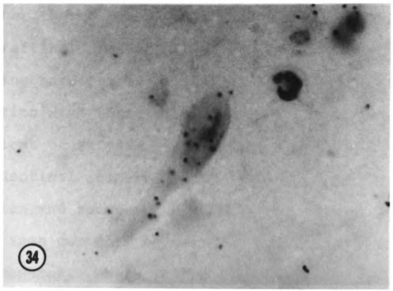
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DISCUSSION

I. Comments on techniques

The excellent success of the grafting technique was more the result of sustained practice than of any particular manipulation. Throughout the experiment, slight changes in the procedure were introduced in order to combat specific problems or eliminate more difficult steps by replacing with simpler ones. The grafting technique outlined above was the result, and, because of several problems yet remaining, it requires a bit of explanation and precaution. The greatest difficulties were encountered in anesthesia. It required a great deal of concentration and effort to simultaneously administer ether to two mice, and at the same time proceed with the cross grafting. Perhaps a solution here would be to use a short-acting barbiturate or similar substance. However, experimentation with these possibilities raised the additional problems of varying responses to the same dosage in otherwise identical animals, and a longer waiting period for both induction and recovery than those for ether.

The same comments hold true for the biopsy and embedding procedures. These techniques were also developed and modified as the experiment progressed. The final product, as outlined, was the best solution to several problems. Taking

a portion of normal skin completely surrounding the graft allowed for more accurate orientation of the tissue within the embedding medium, and consequently, more uniform orientation between sections of all the grafts. The fact that only two out of the last 32 tissues processed contained insufficient graft tissue to provide accurate grain counts bears up the validity and accuracy of the technique, provided the steps are carried out with appropriate care and experience.

The plastic embedding technique was one main reason that such a minute task as separating nuclear from cytoplasmic label could be undertaken. Without the increased resolution, the results could not have been as precise or accurate. Still, the problem of the emulsion being in a different plane of focus remains. It is possible that a more dilute emulsion could have been used so that the silver grains would appear nearer their source; however, the radiation characteristics of tritium (to be discussed below) would also have to be taken into account. At any rate, research involving microscopic analysis of tissue has been enhanced by using the glycol methacrylate embedding material, and these techniques are recommended where precise resolution is desired.

II. Histology of grafts and graft beds

A. Allografts

The appearance described for allografts and graft beds is in agreement with the findings of other investigators. The beginnings of graft destruction are noted grossly after four days with increasing coloration, drying, and shrinkage. Microscopic evidence of graft destruction can be found even earlier in the accumulation of clusters of basophilic cells around the graft vasculature at three to four days (Feldman, 1969), and beginning necrosis of graft epithelium.

The cells seen within the graft bed were the same types as those described in detail by Walker and Goldman (1963) and many other investigators. In allografts, these cells were a combination of fibroblasts, proliferating in the area in order to repair the tissue injury, and lymphocytes which had entered the tissue from the blood and subsequently hypertrophied. As mentioned above, histologic criteria were indeterminate in separating these two populations since there was a continuous range of variation in amounts of basophilia, size of nuclei, and numbers and sizes of nucleoli.

B. Autografts

In autografts, the origin of the basophilic cells was determined to be mainly fibroblasts proliferating locally in response to injury (Walker and Goldman,

1963). The radioautographic evidence from the present experiment supported this conclusion, as discussed below.

The muscle fibers of the panniculus carnosus beneath both allografts and autografts were noted to contain several fibers which were smaller in diameter, more basophilic, and contained a greater proportion of centrally placed nuclei than fibers beneath uninjured skin. The combination of these criteria (central nuclei, basophilia, and small diameter) suggested that these fibers were regenerating (Walker, 1962), probably in response to the injury received at the time of grafting. The infiltration of neutrophils into both allografts and autografts indicated that this was also a non-specific response to injury. The fact that allografts had slightly more neutrophils than autografts was possibly a result of the chemotaxis from the degenerating, necrotic cells of the graft, rather than a cause of the necrosis.

III. Radioautographic labeling

The radioisotope tritium (H^3) was chosen because of its ready applicability to biological systems. Wilson (1966) describes tritium as a beta emitter of very low energy. This fact has at least two implications for biological use. First, its use requires little shielding, and, second, it has great advantages in high resolution radioautography. Furthermore, it is in the class of radioactive compounds with the lowest toxicity.

The fact that tritiated thymidine localizes mainly in nuclei is not surprizing, in view of the fact that the base thymine is incorporated only into DNA and not into RNA. The 18-24 percent of silver grains seen over cytoplasm can be accounted for partially by the background fog and also by the dispersion of the radiation originating from the nucleus. Thus a given beta particle from the nucleus may have been emitted at such an angle as to terminate in the emulsion over adjacent cytoplasm rather than directly over the source of radioactivity. Nevertheless, the 80 percent of label remaining over nuclei gave a sufficiently precise nuclear label so that labeled and unlabeled nuclei were easily discriminated.

On the other hand, the percentages of label over nuclei and cytoplasm for tritiated leucine (Table 3), although having opposite percentages of distribution from those of thymidine, did not take into account the relative cytoplasmic area versus nuclear area. Therefore, a comparison of cytoplasmic and nuclear densities for an animal given only H^3L was also performed. From the data in Table 3, the conclusion can be drawn that tritiated leucine was distributed almost uniformly between cytoplasm and nuclei, with only a slightly greater concentration in the cytoplasm. Therefore the 80 percent of label seen over cytoplasm (Table 3) is accounted for by the fact that cytoplasmic area also is proportionately larger than nuclear area in a given segment of

the graft or graft bed. The nuclear label also indicates that leucine is incorporated into nuclear as well as cytoplasmic proteins.

The fact that amino acids are incorporated into body proteins must be balanced by the possibility that "pools" of amino acids may be present within cells without synthesis into proteins (White, Handler, and Smith, 1964). However, the present experiment deals with the comparison of two cell types within the same animal and their relative, rather than absolute, protein turnovers. Thus the pooling effect, although not necessarily identical in different cell populations, should be similar enough in both populations to eliminate its significance in these results. From the above considerations, and the fact that leucine is incorporated only into the protein fraction of tissue (Reid and Heald, 1970), it was concluded that the H^3leucine distribution reflected the protein production and turnover in the cytoplasm and nuclei of the cells to be discussed.

IV. Radioautography of graft tissue

The relative labeling of graft tissues proved interesting and intriguing. Table 5 indicated that allografts initially had a lower radioactivity than autografts, but continued to increase in radioactivity so that at late time intervals they were more radioactive than the corresponding autografts. In order to eliminate the variation due to differing areas of the graft, the areas counted were chosen

at random between, but not including, the epithelium and the graft bed. The epithelium itself was not counted because its turnover rate is too high to detect any additional migratory or accumulating protein. Also, the epithelium was necrotic on most allografts and activily producing protein in autografts. Thus, elimination of these areas from the counting provided more uniform counts between the two types of grafts, and also a more accurate estimate of protein turnover for similar areas of grafted tissue.

Counts of the grafts for the animal given only H^3T were also performed and indicated that there was only a small contribution of H^3T to either allograft or autograft labeling. This suggested that the labels seen in the other animals were due to the presence of proteins. A wide variety of labeling was seen for the same time interval after grafting (4 days), especially in allografts. It was noted in animals receiving both H^3T and H^3L that these initial allografts were much more radioactive than those on animals receiving only H^3L. But, as stated above, H^3T does not contribute a great deal to the graft labeling, and therefore some other factor(s) must be operating. Likewise, injections of H^3L preceding the biopsy by either 1 or 2 hours did not make a consistant difference in the labeling seen. One consideration is the fact that some of the allografts were not as necrotic as others at the same time intervals, thus could still be producing protein whereas others may have already ceased protein production.

A second possibility lies in the relative degrees of vascularity in the grafts. These grafts were biopsied and H^3L was given on day 4 after grafting, which is the same time as revascularization of grafts takes place (Feldman, 1969). Therefore a given graft which was well vascularized may have been able to take up and incorporate circulating H^3L, whereas a graft not completely vascularized at the same time would not receive a comparable supply of H^3L. These considerations made it imperative to compare grafts only on the same animal, and even so, there is a possibility that grafts on the same animal are not vascularized to the same degree. Nevertheless, the fact that all autografts (except one) were observed to decrease in radioactivity with time within a single animal, while all allografts increased in radioactivity is too consistant to attribute to chance. Furthermore, the allograft tissue of 2 out of 4 animals at the latest time intervals showed a level of radioactivity which exceeded even the highest radioactivity of autografts (at early intervals) for that animal. This last fact is explainable only by an accumulation of labeled protein within allografted tissue which did not occur in autografts. In addition, the accumulation must have originated from outside the graft, since no living cells remained within allografted tissue after 8 days to release any more protein. Comparison of autografts to normal skin showed a parallel decrease in both over time, which is explainable as an expression of the normal turnover

rate of protein produced within these tissues, and would be expected. The origin of the accumulating protein in allografts will be discussed below.

V. Radioautography of graft bed cells

The basophilic cells of the graft beds were separable by the use of radioautography. Walker and Goldman (1963) labeled at differing time intervals relative to grafting, but when H^3T had been given 24 hours before grafting, they noted that only cells from the blood were labeled within the graft bed. When performing cell counts, however, they eliminated cells with less than 5 silver grains over the nucleus and also mentioned the probability that reutilization was occuring to account for the cells which were lightly labeled. Griffiths (1970) also mentioned heavy reutilization in allografts when labeling had been performed to identify short-lived cells. The present study was consistant with these findings. A wide range of nuclear labeling was encountered in graft beds ranging from a few to 20 grains per nucleus (see Appendix B). If reutilization were indeed taking place (and it was obvious that it was by the number of endothelial cells seen with 3 to 4 silver grains over their nuclei) a simple cut off point using a certain minimum number of silver grains per nucleus might well lead to a fibroblast, which had reutilized a considerable amount of label in preparation for division, to be mistaken for a hypertrophied lymphocyte. Consequently it was decided to establish a different criterion

for determining whether a cell had originated from the blood. It was based on the assumption that endothelial cells and fibroblasts would be proliferating at a similar rate and therefore also reutilizing label at a similar rate. In order to include the nuclear size in the criteria, it was based on nuclear grain density (grains per μ^2) rather than an absolute number of grains. The assumption of a similar reutilization rate in these two cell populations is justified because Walker and Goldman (1963) also labeled animals after grafts had been in place for several days and examined the tissue 2 hours after the injection of H^3T. They found fibroblasts and endothelial cells to be the only cell types proliferating in the graft bed area, since both of these populations were heavily labeled. In the present experiment, therefore, the endothelial nuclear label was used as an index of H^3T reutilization. A nuclear label must have been above the 95 percent fiducial limits for endothelial nuclei in order to have definately originated from blood lymphocytes. In addition to increasing the amount of calculations involved, this technique also likely eliminated the cell which may have infiltrated from the blood with a sizable grain density but hypertrophied to twice its size and therefore halved its density. Nevertheless, as noted in Appendix B, Table 18, more than half of the cells counted were still above the cut off point in allografts whereas in autografts the earliest graft had 30 percent and the latest 20 percent of the cells

counted being above the cut off point. These cells in autografts were also very small, corresponding to small lymphocytes rather than the large hypertrophied lymphocytes of the allografts. Once these criteria were established, only data for hypertrophied lymphocytes were tabulated for allografts, while in autografts all large radioactive cells were counted. This additional manipulation was justified, however, when comparing the cytoplasmic protein turnover for the various populations (hypertrophied lymphocytes versus fibroblasts). Table 6, which contains data for a pure population of hypertrophied lymphocytes in allograft beds, indicates more consistant and greater decreases in cytoplasmic densities than does Table 7 which contains the mixed population of all large basophilic cells.

The analysis of cytoplasm to determine the protein turnover rates of these two different populations was carried out for animals receiving both H^3T and H^3L. This also involved a great deal of calculation, but the results were rewarding. Hypertrophied lymphocytes in the allografts initially had a higher cytoplasmic density than did cells in autograft beds. This indicates that they were incorporating H^3L and therefore presumably producing protein at a faster rate than fibroblasts. However, at later intervals, the density for hypertrophied lymphocytes had dropped below the corresponding fibroblast cytoplasmic density. This can be accounted for only by infering that the protein produced had left the cells.

It is easy to hypothesize that the presumed protein production by the hypertrophied lymphocytes is responsible for the accumulating protein of the allograft, and that this solves the mechanism of lymphocyte involvement in graft rejection. However, some additional statistical manipulations indicated that this simple explanation is probably inadequate.

Examination of grafts from animal 3 indicated about 20 unit areas of graft tissue were present in each section of tissue. Assuming this to be true for all time intervals, the absolute increase in number of silver grains seen from early to late intervals can be calculated to be about 500 grains. Likewise it was noted that there were about 80 hypertrophied lymphocytes in each section of graft bed. Assuming that the nuclear labeling criteria accounted for only 50 percent of the lymphocytes actually present, and that the average amount of cytoplasm present per cell on a 2 micra section was 30 μ^2, the absolute number of silver grains released by these cells in labeled protein between the early and late intervals would be 288 grains. Thus the graft appeared to be accumulating more protein than the lymphocytes were producing. There may have been an additional source of protein outside of the hypertrophied lymphocytes which also contributed to the graft rejection process, since there was no such accumulation in autografts. One possible explanation lies in the fact that plasma cells were seen at late intervals in allograft beds (Russell and Monaco, 1965). Presumably these

cells could have incorporated and stored H^3L while it was available, and later released it in a protein against the graft, but this seems unlikely. Another consideration is the amino acid pooling, mentioned earlier. If a pool of labeled amino acid were present in the cytoplasm of a cell at an early interval, it would have been washed out by the fixation process. However, at the later intervals, this pool could have contributed to additional labeled protein production.

An additional source of protein may have come from the sera, which could include a specific substance against allografted tissue. This last possibility is supported by recent *in vivo* findings of systemic humoral antibody against primary skin allografts in mice (Canty and Wunderlich, 1971). However, it is unlikely that this protein could be radioactive, because tritiated leucine would be available for incorporation into tissue proteins for only a few hours after injection on day 4, and this systemic antibody does not appear until day 5. Another explanation for the descrepancy between allograft protein accumulation and graft bed protein production may be the loss of some of the small molecules of the graft bed during fixation.

The present experiment lends no clue to the identity of the substance produced by the hypertrophied lymphocytes of the graft bed other than that it is a protein. This is in accord with *in vitro* studies in which investigators isolated

and analyzed such a protein (Kolb and Granger, 1970 and Williams and Granger, 1969). It might be possible, in some future experiment to collect late skin allografts whose hosts have been injected with H^3leucine and with chromatography or appropriate separation columns, identify the substances which are labeled according to their physical properties. Additional experimentation should also be performed to try to find the source of additional protein production, or loss from the graft bed, since it is likely that graft rejection, being the complex process it is, cannot be completely understood or controlled without such knowledge.

SUMMARY

The essential conclusions of this research are:

(1) lymphocytes from the peripheral blood, having hypertrophied within the graft bed of allografts, produce and release a substance which is probably a protein during the graft rejection process;

(2) there is an accumulation of radioactive label (probably protein) in the dying allograft tissue during the graft rejection process, and

(3) these phenomena (protein release by graft bed cells and protein accumulation in graft tissue) do not occur in autografts.

These findings support the conclusion that lymphocytes are involved in the graft rejection reaction, and suggest that this involvement may be in the form of a protein produced within the graft bed which migrates into the graft and adheres to allogeneic cells.

REFERENCES CITED

REFERENCES CITED

Basten, A., J. F. A. P. Miller, N. L. Warner, and J. Pye 1971 Specific inactivation of thymus-derived (T) and non-thymus-derived (B) lymphocytes by ^{125}I-labeled antigen. Nature (London), 231: 104-106.

Benacerraf, B. and I. Green 1969 Cellular hypersensitivity. Annual Review of Medicine 1969, pp. 141-154.

Billingham, R. E. 1961 Free skin grafting in mammals. Transplantation of Tissues and Cells. Ed. by Billingham and Silvers. The Wistar Institute Press, Philadelphia. pp. 22-23.

Billingham, R. E. 1969 The role of the lymphocyte in transplantation immunity. Anat. Rec., 165: 121-124.

Canty, T. G. and J. R. Wunderlich 1971 Quantitative assessment of cellular and humoral responses to skin and tumor allografts. Transplantation, 11: 111-116.

Cerottini, J. C., A. A. Nordin, and K. T. Brunner 1970 Specific in vitro cytotoxicity of thymus-derived lymphocytes sensitized to alloantigens. Nature (London), 228: 1308-1309.

Cerottini, J. C., A. A. Nordin, and K. T. Brunner 1971 Cellular and humoral response to transplantation antigens. I. Development of alloantibody-forming cells and cytotoxic lymphocytes in the graft-versus-host reaction. J. Exp. Med., 134: 553-564.

Chanana, A. D., E. P. Cronkite, D. D. Joel, L. M. Schiffer, and H. Schnappauf 1969 Studies on lymphocytes. XII. The role of immunologically committed lymphocytes in rejecting skin allografts. Transplantation, 7: 459-467.

Evans, R. and P. Alexander 1971 Rendering macrophages specifically cytotoxic by a factor released from immune lymphoid cells. Transplantation, 12: 227-229.

Feldman, J. D. 1969 Graft rejection. Arch. Int. Med., 123: 713-718.

Feldmann, M. 1972 Cell interactions in the immune response in vitro. II. The requirement for macrophages in lymphoid cell colaboration. J. Exp. Med., 135: 1049-1058.

Fuji, H., M. Zaleski, and F. Milgram 1971 Allogenic nucleated cells as immunogen and target for plaque-forming cells in mice. Transplant. Proc., 3: 852-855.

Giroud, J. P., W. E. Spector, and D. A. Willoughby 1970 Bone marrow and lymph node cells in the rejection of skin allografts in mice. Immunol., 19: 857-863.

Gisler, R. H. and P. Dukor 1972 A three-cell mosaic culture: in vitro response by a combination of pure B- and T-cells with peritoneal macrophages. Cell. Immunol., 4: 341-350.

Golstein, P., H. Wigzell, H. Blomgren, and E. A. J. Svedmyr 1972 Cells mediating specific in vitro cytotoxcity. II. Probable autonomy of thymus-processed lymphocytes (T cells) for killing of allogeneic target cells. J. Exp. Med., 135: 890-906.

Goren, G., G. Berke, and I. Urca 1972 Localization of ^{51}Cr labeled sensitized lymphocytes at the allograft region: a possible diagnostic test in organ transplantation. Surgery, 71: 513-521.

Grant, C. K., G. A. Currie, and P. Alexander 1972 Thymocytes from mice immunized against an allograft render bone marrow cells specifically cytotoxic. J. Exp. Med., 135: 150-164.

Griffiths, A. 1970 Skin transplantation and the types of lymphocytes involved in rejection. J. Anat., 107: 194.

Hamilton, D. N. H., J. E. Castro, and J. M. Gaugas 1971 Cell-mediated and humoral mechanisms in allograft and xenograft skin rejection. Br. J. Surg., 58: 858-859.

Hayry, P. and V. Defendi 1970 Mixed lymphocyte cultures produce effector cells: model in vitro for allograft rejection. Science, 168: 133-135.

Jakobisiak, M. 1971 Quantitative data concerning the development of the cellular infiltration of skin allograft in mice. Transplantation, 12: 364-367.

Kolb, W. P. and G. A. Granger 1970 Lymphocyte in vitro cytotoxicity: characterization of mouse lymphotoxin. Cell. Immunol., 1: 122-132.

Kramer, J. J. and G. A. Granger 1972 In vitro induction and release of a cell toxin by immune C57Bl/6 mouse peritoneal macrophages. Cell. Immunol., 3: 88-100.

Lewis, A. E. 1966 Biostatistics, Reinhold Publishing Corporation, New York, p. 213.

Liem, P. L. and C. Jerusalem 1970 Blood supply of allogeneic skin transplants before and during graft rejection. Act. Morph. N., 7: 356-357.

Lonai, P. and M. Feldman 1971_a Cooperation of lymphoid cells in an in vitro graft reaction system. II. The "bone marrow-derived" cell. Transplantation, 11: 446-456.

Lonai, P. and M. Feldman 1971_b Studies on the effect of macrophages in an in vitro graft reaction system. Immunol., 21: 861-867.

Lykke, A. W. J. and R. Cummings 1970 Increased vascular permeability in the primary allograft response in the skin of the rat. J. Pathol., 101: 319-327.

Melchers, F. 1970 Biosynthesis of the carbohydrate portion of immunoglobulins. Kinetics of synthesis and secretion of H^3-leucine, H^3-galactose, and H^3-mannose labeled myeloma protein by two plasma-cell tumours. Biochem. J., 119: 765-772.

Miller, J. F. A. P., A. Basten, J. Sprent, and C. Cheers 1971_a Interaction between lymphocytes in immune responses. Cell. Immunol., 2: 469-495.

Miller, J. F. A. P., J. Sprent, A. Basten, N. L. Warner, J. C. S. Breitner, G. Rowland, J. Hamilton, H. Silver, and W. J. Martin 1971_b Cell-to-cell interaction in the immune response. VII. Requirement for differentiation of thymus-derived cells. J. Exp. Med., 134: 1266-1284.

Miller, J. F. A. P., K. T. Brunner, J. Sprent, P. J. Russell, and G. F. Mitchell 1971_c Thymus-derived cells as killer cells in cell-mediated immunity. Transplan. P., 3: 915-917.

Moller, E. and W. Lapp 1969 Cytotoxic effects in vitro by lymphoid cells from specifically tolerant animals. Immunol., 16: 561-566.

Osmond, D. G. 1969 The non-thymic origin of lymphocytes. Anat. Rec., 165: 109-112.

Peter, H. H. and J. D. Feldman 1972 Cell-mediated cytotoxicity during rejection and enhancement of allogeneic skin grafts in rats. J. Exp. Med., 135: 1301-1315.

Reid, R. J. and P. J. Heald 1970 Uptake of H^3-leucine into proteins of rat uterus during early pregnancy. Biochem. Biophys. Acta, 204: 278-279.

Russell, P. S. and A. P. Monaco 1965 The Biology of Tissue Transplantation. Little, Brown, and Company, Boston, P. 18.

Salvin, S. B., S. Sell, and J. Nishio 1971 Activity in vitro of lymphocytes and macrophages in delayed hypersensitivity. J. Immunol., 107: 655-662.

Slonecker, C. E. 1969 H^3-leucine incorporation in antigenically stimulated rat popliteal lymph node cells. Anat. Rec., 165: 363-378.

Tilney, N. L. and J. L. Gowans 1971 The sensitization of rats by allografts transplanted to alymphatic pedicels of skin. J. Exp. Med., 133: 951-962.

Wagner, H., A. W. Harris, and M. Feldmann 1972 Cell-mediated immune response in vitro. II. Role of thymus and thymus-derived lymphocytes. Cell. Immunol. 4: 39-50.

Walker, B. E. 1959 Radioautographic observations on regeneration of transitional epithelium. Tex. Rep. Biol. Med., 17: 375-384.

Walker, B. E. 1962 A radioautographic study of muscle regeneration in dystrophic mice. Am. J. Path., 41: 41-53.

Walker, B. E. and A. S. Goldman 1963 Thymidine-H^3 radioautography of skin grafts in mice. Tex. Rep. Biol. Med., 21: 425-441.

Walker, B. E., R. D. Yates, and D. Duncan 1964 Cell morphology in tissue underlying skin grafts. Anat. Rec., 149: 651-670.

White, A., P. Handler, and E. L. Smith 1964 Principles of Biochemistry, 3rd ed., McGraw-Hill Book Company, New York, p. 507.

Williams, T. W. and G. A. Granger 1969 Lymphocyte in vitro cytotoxicity: mechanism of lymphotoxin-induced target cell destruction. J. Immunol., 102: 911-918.

Wilson, B. J. 1966 The Radiochemical Manual, 2nd ed., The Radiochemical Centre, Amersham, p. 42.

Yunis, E. J., O. Stutman, and R. A. Good 1971 Thymus, immunity, and autoimmunity. Ann. N. Y. Acad. Sci., 183: 205-220.

Zatz, M. M. and E. M. Lance 1971 The distribution of ^{51}Cr labeled lymphocytes into antigen stimulated mice. Lymphocyte trapping. J. Exp. Med., 134: 224-241.

APPENDICES

APPENDIX A

Appendix A. Comprehensive data for graft labeling

This appendix lists the actual grains counted for randomly selected areas (2500 μ^2) of the graft tissue. Areas were located within the graft tissue between the epidermis and graft bed. This would include the dermis and panniculus adiposus. Means were calculated for the ten areas from each graft. Standard deviations were calculated using the formula $sd = \sqrt{\Sigma(\bar{X} - X)^2/(n - 1)}$. Grafts of the same type for the same animal at different time intervals were compared for statistically significant changes by applying the t test using the formula $t = (\bar{X}_1 - \bar{X}_2)/s' \sqrt{(1/n_1) + (1/n_2)}$, where $s' = \sqrt{(n_1 - 1)V_1 + (n_2 - 1)V_2/(n_1 - 1) + (n_2 - 1)}$ or, because $n_1 = n_2 = 10$ in this case, $s' = \sqrt{(V_1 + V_2)/2}$ and $t = (\bar{X}_1 - \bar{X}_2)/s'(.45)$. The appropriate tables were then consulted (Lewis, 1966) for p values.

Table 8. Grain counts of allograft tissue from animal 9 (leucine).

Days after grafting	4.1	8	10
grain counts (grains/2500μ^2)	17	38	54
	15	44	46
	19	46	45
	17	43	40
	22	36	46
	16	31	40
	20	35	38
	18	36	41
	21	38	37
	23	43	45
Mean	18.6	38.9	43.2
Standard deviation	2.7	4.6	5.0
t		11.95	2.00
p		$<.001$	$<.10$

Table 9. Grain counts of autograft tissue from animal 9 leucine).

Days after grafting	4.1	8	10
grain counts (grains/2500μ^2)	insufficient tissue available	47	39
		40	38
		46	37
		44	31
		41	40
		44	34
		42	41
		46	37
		45	43
		51	38
Mean		44.6	37.8
Standard deviation		3.2	3.5
t			4.48
p			$<.001$

Table 10. Grain counts of allograft tissue from animal 10 (leucine).

Days after grafting	4.2	5	6
grain counts (grains/2500μ^2)	18	38	58
	11	36	52
	12	22	53
	16	30	48
	12	37	67
	14	27	61
	19	28	53
	14	23	68
	18	33	60
	11	37	57
Mean	14.5	31.1	57.6
Standard deviation	3.1	6.0	6.5
t		7.77	9.44
p		$<.001$	$<.001$

Table 11. Grain counts of autograft tissue from animal 10 (leucine).

Days after grafting	4.2	5	6
grain counts (grains/2500μ^2)	24	40	37
	24	38	33
	33	43	33
	32	38	38
	33	39	43
	38	35	39
	35	36	35
	32	33	35
	34	39	31
	33	35	38
Mean	31.8	37.6	36.2
Standard deviation	4.5	2.9	3.5
t		3.42	0.96
p		$<.005$	$<.50$

Table 12. Grain counts of allografts from animal 4 (H^3T and H^3L).

Days after grafting	4.1	8	10
grain counts (grains/2500μ^2)	37	insufficient tissue available	47
	44		53
	41		41
	43		43
	33		48
	50		49
	39		53
	48		51
	42		47
	41		44
Mean	38.8		47.6
Standard deviation	5.9		4.1
t	3.86		
p	$<.005$		

Table 13. Grain counts of autografts from animal 4 (H^3T and H^3L).

Days after grafting	4.1	8	10
grain counts (grains/2500μ^2)	61	36	30
	51	40	35
	60	41	31
	49	44	30
	50	44	34
	48	38	28
	54	38	31
	47	34	36
	54	36	38
	54	33	32
Mean	52.8	38.4	32.5
Standard deviation	4.8	3.8	3.1
t	7.39	3.74	
p	$<.001$	$<.005$	

Table 14. Grain counts of allografts from animal 3 (H^3T and H^3L).

Days after grafting	4.1	5	6
grain counts (grains/2500μ^2)	40	52	61
	47	56	68
	51	44	74
	39	51	63
	37	48	63
	43	47	67
	45	49	65
	41	52	71
	40	56	70
	37	50	65
Mean	42.0	50.5	66.7
Standard deviation	4.5	3.8	4.1
t		4.54	9.14
p		$<.001$	$<.001$

Table 15. Grain counts of autografts from animal 3 (H^3T and H^3L).

Days after grafting	4.1	5	6
grain counts (grains/2500μ^2)	55	35	35
	58	38	36
	65	42	38
	62	37	40
	53	37	29
	54	32	30
	50	31	38
	51	37	35
	52	33	35
	51	34	31
Mean	55.1	35.7	34.7
Standard deviation	5.0	3.3	3.7
t		10.14	0.64
p		$<.001$	$>.50$

APPENDIX B

Appendix B. Nuclear labeling in animals receiving H^3T

In animals receiving only H^3T, or both H^3L and H^3T, nuclear labeling characteristics were used to identify hypertrophied lymphocytes of the graft beds. Endothelial cells were used as an index of reutilization of the H^3T label, and it was assumed that fibroblasts would reutilize to about the same degree. Therefore, taking the mean nuclear density of endothelial cells and adding twice its standard deviation produces a cut off point; and cells with nuclear densities higher than that point are assumed to be hypertrophied lymphocytes originating from the blood. Data for endothelial cell nuclei, cut off points, hypertrophied lymphocyte nuclei (allografts), and basophilic nuclei (autografts) are presented in this appendix. All nuclear areas are rounded to the nearest 5 μ^2. Symbols used are as follows:

\# = grain count

μ^2 = area in square micra

$\#/\mu^2$ = grain density

4.1 = one hour following H^3L injection on day 4

4.2 = two hours following H^3L injection on day 4

Table 16. Endothelial nuclear counts for animal 7 (thymidine).

Days after grafting	4			6			8			10		
	#	μ^2	#/μ^2	#	μ^2	#/μ^2	#	μ^2	#/μ^2	#	μ^2	#/μ^2
Allograft endothelial counts	6	25	.24	4	25	.18	2	10	.20	3	15	.20
	7	30	.23	3	20	.15	3	20	.15	2	20	.10
	4	20	.20	3	15	.20	4	25	.16	1	20	.05
	6	25	.24	2	10	.20	2	20	.10	3	15	.20
	4	25	.16	3	15	.20	3	15	.20	2	15	.13
	6	25	.24	3	15	.20	3	15	.20	1	15	.07
	4	20	.20	4	25	.16	2	10	.20	1	10	.10
	5	20	.25				1	10	.10	1	10	.10
	2	10	.20							2	15	.13
	6	25	.24							1	10	.10
	7	25	.28									
	4	15	.27									
	5	30	.17									
Mean density		.22			.18			.16			.12	
Standard deviation		.04			.02			.04			.05	
Cut off point		.30			.22			.24			.22	
	#	μ^2	#/μ^2	#	μ^2	#/μ^2	#	μ^2	#/μ^2	#	μ^2	#/μ^2
Autograft endothelial counts	3	20	.15	3	15	.20	5	30	.17	4	30	.13
	4	20	.20	4	20	.20	3	25	.12	3	20	.15
	7	15	.20	6	30	.20	4	20	.20	4	20	.20
	2	20	.10	7	40	.18	5	20	.25	2	20	.10
	7	40	.18	4	30	.13	5	25	.20	3	20	.15
	3	25	.12	3	20	.15	3	20	.15	2	25	.08
	7	30	.23	5	40	.13	4	20	.20	3	25	.12
	5	25	.20	3	20	.15	3	30	.10	4	30	.13
				3	20	.15	2	20	.10			
							3	25	.12			
Mean density		.17			.17			.16			.13	
Standard deviation		.04			.03			.05			.04	
Cut off point		.25			.23			.26			.21	

Table 17. Basophilic cell nuclear counts of allografts from animal 7 (thymidine).

Days after grafting	4			6			8			10		
	#	μ^2	#/μ^2	#	μ^2	#/μ^2	#	μ^2	#/μ^2	#	μ^2	#/μ^2
	5	50	.10	4	40	.10	5	60	.08	3	30	.10
	9	50	.18	5	40	.13	4	40	.10	5	50	.10
	12	60	.20	5	40	.13	5	50	.10	4	25	.16
	8	40	.20	4	25	.16	4	30	.13	5	30	.17
	13	60	.22	5	30	.17	7	45	.16	10	40	.25
	7	30	.23	5	30	.17	6	35	.17	10	40	.25
	17	65	.26	7	35	.20	10	45	.22	13	50	.26
	11	40	.27	7	30	.23	6	25	.23	11	40	.28
Basophilic cell	12	40	.30	10	40	.25	7	30	.23	6	20	.30
nuclei	6	20	.30	8	30	.27	7	30	.23	8	25	.32
(in order of	11	35	.31	8	30	.27	12	50	.24	9	25	.36
increasing density)	10	30	.33	7	25	.28	4	15	.27	12	30	.40
	9	25	.36	7	25	.28	10	30	.33	11	25	.44
	11	30	.37	7	25	.28	9	25	.36			
	12	30	.40	8	25	.32	8	20	.40			
	12	30	.40	10	30	.33	12	30	.40			
	21	50	.42	10	30	.33	12	25	.48			
	15	35	.43	9	25	.36	18	35	.51			
	14	30	.47	10	25	.40	13	25	.52			
	17	30	.57	10	25	.40	17	30	.57			
Cut off point (from Table 16)		.30			.22			.24			.22	
% above cut off point		50			64			45			69	

Table 18. Basophilic cell nuclear counts of autografts from animal 7 (thymidine).

Days after grafting	4			6			8			10		
	#	μ^2	#/μ^2	#	μ^2	#/μ^2	#	μ^2	#/μ^2	#	μ^2	#/μ^2
Basophilic cell nuclei (in order of increasing density)	4	30	.13	5	50	.10	6	50	.12	4	30	.13
	7	50	.14	4	30	.13	12	80	.15	5	40	.13
	9	50	.18	6	40	.15	8	50	.16	6	40	.15
	8	40	.20	5	30	.17	4	25	.16	6	40	.15
	8	40	.20	5	30	.17	8	50	.16	5	30	.17
	6	30	.20	8	40	.20	10	60	.17	6	35	.17
	7	35	.20	10	50	.20	6	35	.17	7	40	.18
	12	60	.20	8	40	.20	5	30	.17	7	40	.18
	9	40	.22	7	35	.20	7	40	.18	12	60	.20
	9	40	.22	8	40	.20	7	40	.18	5	25	.20
	12	50	.24	9	40	.22	11	60	.18	6	30	.20
	10	40	.25	11	50	.22	10	50	.20	8	40	.20
	10	35	.29	9	30	.30	8	30	.27	6	30	.20
	13	35	.37	17	50	.34	9	30	.30	8	40	.20
	12	30	.40	12	30	.40	12	40	.30	10	50	.20
	12	30	.40	13	30	.43	9	20	.45	5	20	.25
	13	25	.52							5	20	.25
	16	30	.53							8	25	.32
										8	25	.32
										12	30	.40
Cut off point (from Table 16)		.25			.23			.26			.21	
% above cut off point		30			25			25			20	

Table 19. Endothelial nuclear counts for animal 4 (thymidine and leucine).

Days after grafting	4.1			8			10		
	#	μ^2	#/μ^2	#	μ^2	#/μ^2	#	μ^2	#/μ^2
Allograft endothelial counts	2	10	.20	2	20	.10	3	20	.15
	5	25	.20	2	30	.07	4	20	.20
	2	10	.20	2	20	.10	4	25	.16
	9	50	.18	3	15	.20	2	20	.10
	4	20	.20	5	25	.20	3	15	.20
	4	25	.16	3	20	.15	5	25	.20
	7	30	.23	3	30	.10	5	25	.20
	4	20	.20	3	25	.15	4	20	.20
	5	20	.25	4	20	.20	5	20	.25
	5	20	.25	2	20	.12	3	20	.15
Mean density		.21			.14			.18	
Standard deviation		.03			.05			.04	
Cut off point		.27			.24			.26	
	#	μ^2	#/μ^2	#	μ^2	#/μ^2	#	μ^2	#/μ^2
Autograft endothelial counts	4	20	.20	4	20	.20	4	40	.10
	2	15	.13	4	30	.13	3	30	.10
	3	20	.15	2	20	.10	8	50	.16
	2	20	.10	2	15	.13	6	40	.15
	3	20	.15	3	20	.15	2	20	.10
	3	15	.20	3	20	.15	3	20	.15
	7	30	.23	4	20	.20	3	20	.15
	6	30	.20	3	25	.12	2	20	.10
	3	20	.15	4	25	.16	3	30	.10
	4	25	.16	2	20	.10	3	25	.12
Mean density		.17			.14			.12	
Standard deviation		.04			.03			.03	
Cut off point		.25			.20			.18	

Table 20. Basophilic cell nuclear counts of allografts from animal 4 (H^3T and H^3L).

Days after grafting	4.1			8			10		
	#	μ^2	#/μ^2	#	μ^2	#/μ^2	#	μ^2	#/μ^2
Hypertrophied lymphocyte nuclei (in order of increasing density)	11	40	.28	10	40	.25	8	30	.27
	14	50	.28	5	20	.25	14	50	.28
	11	40	.28	10	40	.25	14	50	.28
	7	25	.28	13	50	.26	7	25	.28
	7	25	.28	8	30	.27	7	25	.28
	6	20	.30	8	30	.27	7	25	.28
	12	40	.30	7	25	.28	6	20	.30
	9	30	.30	11	40	.28	6	20	.30
	16	50	.32	12	40	.30	8	25	.32
	10	30	.33	12	40	.30	8	25	.32
	5	15	.33	6	20	.30	10	30	.33
	17	50	.34	12	40	.30	14	40	.35
	9	25	.36	12	40	.30	9	25	.36
	13	25	.37	11	35	.31	9	25	.36
	12	30	.40	8	25	.32	18	50	.36
	8	20	.40	10	30	.33	9	25	.36
	20	50	.40	10	30	.33	9	25	.36
	17	40	.43	18	50	.36	18	50	.36
	13	30	.43	15	40	.38	8	20	.40
	22	50	.44	12	30	.40	11	25	.44
Cut off point (from Table 19)		.27			.24			.26	
% above cut off point		100			100			100	

Table 21. Basophilic cell nuclear counts of autografts from animal 4 (H^3T and H^3L).

Days after grafting	4.1			8			10		
	#	μ^2	#/μ^2	#	μ^2	#/μ^2	#	μ^2	#/μ^2
Basophilic cell nuclei (in order of increasing density)	6	50	.12	4	40	.10	3	30	.10
	6	40	.15	5	40	.13	8	80	.10
	8	50	.16	5	40	.13	7	60	.12
	8	50	.16	4	30	.13	6	50	.12
	5	30	.17	5	40	.13	5	40	.13
	7	40	.18	6	40	.15	9	70	.13
	9	50	.18	6	40	.15	5	35	.14
	7	40	.18	6	40	.15	7	50	.14
	9	50	.18	5	30	.17	12	80	.15
	10	50	.20	6	35	.17	6	40	.15
	4	20	.20	5	30	.17	7	45	.16
	8	40	.20	7	40	.18	13	80	.16
	8	40	.20	9	50	.18	8	50	.16
	10	50	.20	7	40	.18	7	45	.16
	5	25	.20	8	40	.20	8	50	.16
	7	30	.23	5	25	.20	13	80	.16
	10	40	.25	10	50	.20	7	40	.18
	11	40	.28	7	30	.23	7	40	.18
	9	25	.36	6	25	.24	6	30	.20
	8	20	.40	8	25	.32	10	50	.20
Cut off point (from Table 19)		.25			.20			.18	
% above cut off point		15			15			10	

Table 22. Endothelial nuclear counts for animal 3 (thymidine and leucine).

Days after grafting	4.2			5			6		
	#	μ^2	#/μ^2	#	μ^2	#/μ^2	#	μ^2	#/μ^2
Allograft endothelial counts	5	25	.20	6	30	.20	3	20	.15
	5	25	.20	5	25	.20	3	20	.15
	6	30	.20	4	25	.16	4	25	.16
	6	40	.15	4	20	.20	4	20	.20
	5	30	.17	5	30	.17	4	20	.20
	9	50	.18	4	25	.16	4	25	.16
	3	20	.15	4	20	.20	3	20	.15
	4	25	.16	3	20	.15	2	20	.10
	5	30	.17	3	20	.15	2	15	.13
	4	25	.16	4	25	.16	3	20	.15
Mean density		.17			.18			.16	
Standard deviation		.02			.02			.03	
Cut off point		.21			.22			.22	
	#	μ^2	#/μ^2	#	μ^2	#/μ^2	#	μ^2	#/μ^2
Autograft endothelial counts	5	25	.20	3	20	.15	5	25	.20
	6	30	.20	3	20	.15	3	20	.15
	6	25	.24	2	20	.10	4	20	.20
	4	20	.20	3	25	.12	4	25	.16
	3	20	.15	3	20	.15	3	20	.15
	4	25	.16	6	30	.20	5	25	.20
	5	25	.20	6	25	.24	5	20	.25
	3	15	.20	5	30	.17	4	20	.20
	6	25	.24	6	25	.24	3	15	.20
	3	15	.20	5	20	.25	2	10	.20
Mean density		.20			.18			.19	
Standard deviation		.03			.05			.03	
Cut off point		.26			.28			.25	

Table 23. Basophilic cell nuclear counts of allografts from animal 3 (H^3T and H^3L).

Days after grafting	4.2			5			6		
	#	μ^2	#/μ^2	#	μ^2	#/μ^2	#	μ^2	#/μ^2
	9	40	.23	9	40	.23	7	30	.23
	9	40	.23	7	30	.23	7	30	.23
	7	30	.23	9	40	.23	7	30	.23
	7	30	.23	9	40	.23	9	40	.23
	9	40	.23	9	40	.23	9	40	.23
	6	25	.24	7	30	.23	6	25	.24
	10	40	.25	6	25	.24	10	40	.25
Hypertrophied	8	30	.27	10	40	.25	10	40	.25
lymphocyte nuclei	8	30	.27	10	40	.25	8	30	.27
(in order of	9	30	.30	8	30	.27	8	30	.27
increasing density)	9	30	.30	8	30	.27	8	30	.27
	8	25	.32	8	30	.27	8	30	.27
	10	30	.33	7	25	.28	9	30	.30
	10	30	.33	7	25	.28	9	30	.30
	10	30	.33	7	25	.28	8	25	.32
	10	30	.33	9	30	.30	8	25	.32
	10	30	.33	8	25	.32	10	30	.33
	12	30	.40	10	30	.33	10	30	.33
	12	30	.40	10	30	.33	9	25	.36
	13	30	.43	11	30	.37	12	30	.40
Cut off point (from Table 22)		.21			.22			.22	
% above cut off point		100			100			100	

Table 24. Basophilic cell nuclear counts of autografts from animal 3 (H^3T and H^3L).

Days after grafting	4.2			5			6		
	#	μ^2	#/μ^2	#	μ^2	#/μ^2	#	μ^2	#/μ^2
	8	60	.13	7	50	.14	8	50	.16
	7	50	.14	8	50	.16	8	50	.16
	8	50	.16	10	60	.17	5	30	.17
	8	50	.16	9	50	.18	5	30	.17
	10	60	.17	7	40	.18	5	30	.17
	5	30	.17	9	50	.18	9	50	.18
	7	40	.18	7	40	.18	8	40	.20
Basophilic cell	9	50	.18	8	40	.20	10	50	.20
nuclei	14	80	.18	6	30	.20	5	25	.20
(in order of	5	25	.20	8	40	.20	6	30	.20
increasing density)	8	40	.20	12	60	.20	7	35	.20
	6	30	.20	10	50	.20	8	40	.20
	10	50	.20	8	40	.20	7	35	.20
	8	40	.20	6	30	.20	6	30	.20
	9	40	.23	8	40	.20	7	30	.23
	7	30	.23	7	30	.23	7	30	.23
	8	30	.27	8	30	.27	9	40	.23
	8	30	.27	9	30	.30	10	40	.25
	11	40	.28	12	40	.30	8	30	.27
	12	40	.30	10	30	.33	10	30	.33
Cut off point (from Table 22)		.26			.28			.25	
% above cut off point		20			15			10	

APPENDIX C

Appendix C. Cytoplasmic labeling in graft beds

In animals receiving both H^3T and H^3L, hypertrophied lymphocytes of allografts were observed to have an initially higher cytoplasmic density than the basophilic cells in the autograft beds. At late intervals, allograft cytoplasmic density declined to below that for autografts. This appendix presents the data from which these conclusions were drawn. All cytoplasmic areas were rounded to the nearest 5 μ^2. Symbols used are as follows:

#=grain count

μ^2=area in square micra

$\#/\mu^2$=grain density

4.1=one hour following H^3L injection on day 4

4.2=two hours following H^3L injection on day 4

These cytoplasmic data are for the same cells whose nuclear data are given in Appendix B, although not in the same order.

Table 25. Cytoplasmic labeling in allografts of animal 4.

Days after grafting	4.1			8			10		
	#	μ^2	#/μ^2	#	μ^2	#/μ^2	#	μ^2	#/μ^2
Hypertrophied lymphocyte cytoplasmic counts	10	40	.25	6	25	.24	2	15	.13
	3	20	.15	2	15	.13	3	20	.15
	8	35	.23	3	15	.20	1	10	.10
	8	30	.27	3	20	.15	3	20	.15
	5	15	.33	2	10	.20	3	20	.15
	13	60	.22	2	15	.13	2	20	.10
	10	35	.29	2	20	.10	4	30	.13
	12	30	.40	2	20	.10	5	30	.17
	5	20	.25	3	25	.12	2	20	.10
	6	25	.24	2	20	.10	5	25	.20
	4	15	.27	3	20	.15	3	25	.12
	11	35	.31	6	35	.17	2	20	.10
	6	30	.20	6	30	.20	8	20	.11
	6	25	.24	4	25	.16	7	40	.18
	11	50	.22	3	30	.10	4	50	.08
	7	30	.23	2	20	.10	3	25	.12
	13	60	.22	4	30	.13	3	20	.15
	4	20	.20	5	40	.13	5	30	.17
	8	30	.27	2	10	.20	14	100	.14
	4	15	.27	5	30	.17	9	50	.18
Mean density		.25			.15			.14	
Standard deviation		.05			.04			.03	
t value			7.92			.917			
p			$<.001$			$<.50$			

Table 26. Cytoplasmic labeling in autografts of animal 4.

Days after grafting	4.1			8			10		
	#	μ^2	#/μ^2	#	μ^2	#/μ^2	#	μ^2	#/μ^2
Basophilic cell cytoplasmic counts	4	25	.16	7	40	.18	4	40	.10
	3	30	.10	6	30	.20	5	40	.13
	4	30	.13	5	30	.17	4	30	.13
	2	10	.20	2	10	.20	3	15	.20
	6	30	.20	3	20	.15	5	30	.17
	2	10	.20	3	25	.12	7	40	.18
	3	30	.10	2	10	.20	6	30	.20
	3	25	.12	2	10	.20	2	10	.20
	6	50	.12	3	15	.20	2	15	.13
	4	25	.16	4	20	.20	3	20	.15
	11	100	.11	5	25	.20	3	20	.15
	1	10	.10	7	50	.14	3	30	.10
	5	40	.13	5	40	.13	3	30	.10
	3	30	.10	4	20	.20	3	15	.20
	4	30	.13	2	10	.20	10	80	.13
	5	40	.13	2	15	.13	9	50	.18
	3	20	.15	2	15	.13	8	40	.20
	5	30	.17	3	15	.20	7	40	.18
	6	30	.20	3	20	.15	8	50	.16
	4	25	.16	4	20	.20	6	40	.15
Mean density		.14			.17			.16	
Standard deviation		.04			.03			.03	
t value			2.88			0.96			
p			$<.01$			$<.50$			

Table 27. Cytoplasmic labeling in allografts of animal 3.

Days after grafting	4.2			5			6		
	#	μ^2	#/μ^2	#	μ^2	#/μ^2	#	μ^2	#/μ^2
	4	20	.20	4	30	.13	6	50	.12
	6	25	.24	5	30	.17	4	40	.10
	2	20	.10	8	40	.20	2	20	.10
	3	15	.20	3	20	.15	7	60	.12
	6	30	.20	4	25	.16	6	30	.20
	4	25	.16	4	30	.13	3	20	.15
	4	20	.20	3	20	.15	3	25	.12
Hypertrophied	6	25	.24	3	25	.12	6	35	.17
lymphocyte	3	20	.15	4	25	.16	5	40	.13
cytoplasmic	5	30	.17	2	15	.13	3	25	.12
counts	2	10	.20	3	25	.12	3	20	.15
	7	40	.18	4	30	.13	3	25	.12
	4	25	.16	5	30	.17	5	40	.13
	7	40	.18	6	40	.15	4	30	.13
	3	15	.20	2	20	.10	3	20	.15
	7	30	.30	6	30	.20	4	20	.20
	4	20	.20	5	30	.17	3	20	.15
	2	10	.20	3	25	.12	2	20	.10
	3	20	.15	3	15	.20	3	25	.12
	4	20	.20	4	30	.13	2	20	.10
Mean density		.19			.15			.13	
Standard deviation		.03			.03			.03	
t value			4.26			2.25			
p			$<.001$			$<.05$			

Table 28. Cytoplasmic labeling in autografts of animal 3.

Days after grafting	4.2			5			6		
	#	μ^2	#/μ^2	#	μ^2	#/μ^2	#	μ^2	#/μ^2
Basophilic cell cytoplasmic counts	5	50	.10	3	20	.15	3	20	.15
	4	25	.16	3	20	.15	2	15	.13
	3	20	.15	4	30	.13	3	25	.12
	2	10	.20	4	25	.16	4	30	.13
	3	20	.15	4	20	.20	2	20	.10
	5	40	.13	9	50	.18	5	30	.17
	3	25	.12	7	40	.18	2	15	.13
	5	30	.17	3	30	.10	9	50	.18
	3	30	.10	4	30	.13	5	30	.17
	4	20	.20	4	20	.20	7	40	.18
	3	20	.15	3	25	.12	4	20	.20
	5	40	.13	4	25	.16	4	25	.16
	3	30	.10	5	30	.17	5	25	.20
	4	20	.20	4	30	.13	4	30	.13
	2	10	.20	3	25	.12	3	20	.15
	5	30	.17	3	20	.15	2	15	.13
	3	25	.12	2	10	.20	5	30	.17
	3	20	.15	2	15	.13	2	15	.13
	5	40	.13	1	10	.10	4	20	.20
	6	50	.12	3	20	.15	4	25	.16
Mean density		.15			.15			.15	
Standard deviation		.03			.03			.03	
t value			no difference			no difference			
p			$>.50$			$>.50$			

APPENDIX D

Appendix D. Distribution of isotope labels.

In animals given only H^3T, the majority of silver grains were located over nuclei, whereas in those animals given only H^3L most of the grains localized over cytoplasm. Random 2500 μ^2 areas of graft tissue, graft beds, or normal dermis were analyzed and the grains located over cytoplasm and over nuclei were tabulated separately and are listed in this appendix. Abbreviations used are as follows:

nuc.=nucleus

cyt.=cytoplasm

#=number of silver grains

μ^2=area in square micra

%=percent of label

Table 29. Leucine label distribution

Allografts						Autografts					
Animal 9											
		days after grafting						days after grafting			
4.1		8		10		4.1		8		10	
nuc.	cyt.	nuc.	cyt.	nuc.	cyt.	nuc.	cyt.	nuc.	cyt.	nuc.	cyt.
13	58	12	51	21	66	13	64	13	63	14	53
# 8	40	14	53	20	75	8	76	13	76	14	77
12	33	12	84	14	77	13	54	20	66	20	83
%20	80	17	83	20	80	15	85	18	82	18	82
Animal 10											
		days after grafting						days after grafting			
4.2		5		6		4.2		5		6	
nuc.	cyt.	nuc.	cyt.	nuc.	cyt.	nuc.	cyt.	nuc.	cyt.	nuc.	cyt.
14	68	11	48	32	98	24	75	13	53	17	112
#12	68	17	53	20	83	18	46	24	69	20	97
5	47	17	84	19	67	12	65	12	54	9	74
%14	86	20	80	22	78	22	78	22	78	14	86

Table 30. Nuclear and cytoplasmic grain densities in animal 9 (leucine).

Allografts days after grafting						Autografts days after grafting					
4.1		8		10		4.1		8		10	
Cytoplasm											
#	μ^2	#	μ^2	#	μ^2	#	μ^2	#	μ^2	#	μ^2
4	20	3	10	5	40	4	20	5	25	5	30
4	15	6	20	4	20	4	20	4	20	4	20
5	15	4	25	3	20	12	70	7	30	3	20
5	20	7	40	4	25	8	40	3	20	4	30
6	25	4	25	7	60	9	60	2	10	7	35
6	20	4	25	4	25	5	30	4	25	4	25
5	30	5	25	4	20	9	40	4	20	8	50
6	20	3	20	3	20	7	30	6	30	5	30
10	40	4	20	3	20	7	30	4	25	6	30
3	10	2	10	5	50	7	35	5	25	4	20
$\#/\mu^2$	.25		.19		.14		.19		.19		.17
Nuclei											
#	μ^2	#	μ^2	#	μ^2	#	μ^2	#	μ^2	#	μ^2
7	50	3	20	3	25	5	30	5	25	4	25
7	60	3	25	5	40	9	50	6	40	6	40
13	100	4	50	3	25	6	40	6	50	6	40
9	60	5	35	3	30	9	40	6	40	4	25
6	40	5	50	6	25	9	50	5	40	5	30
$\#/\mu^2$	.14		.11		.12		.18		.15		.16

Table 31. Thymidine label distribution (animal 7).

	days after grafting							
	4		6		8		10	
	Allografts							
	nuc.	cyt.	nuc.	cyt.	nuc.	cyt.	nuc.	cyt.
	63	16	56	20	33	9	42	13
	53	18	64	21	24	8	50	14
#	49	12	66	22	33	7	25	6
	57	23	74	19	53	11	55	12
	77	20	52	15	45	7	60	16
%	77	23	76	24	81	19	79	21
	Autografts							
	70	17	35	9	39	11	60	16
	75	27	43	19	57	14	29	6
#	56	14	62	15	56	12	40	13
	81	15	68	20	50	12	29	8
	49	15	42	15	60	20	36	8
%	79	21	76	24	82	18	79	21