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PASSIVE TRANSFER OF HOMOGRAFT
SENSITIVITY IN GUINEA PIGS

by
George Edward Lowke

A Dissertation Submitted to the Faculty of the
DEPARTMENT OF MICROBIOLOGY AND MEDICAL TECHNOLOGY
In Partial Fulfillment of the Requirements
For the Degree of
DOCTOR OF PHILOSOPHY
WITH A MAJOR IN MICROBIOLOGY
In the Graduate College
THE UNIVERSITY OF ARIZONA

1969
I hereby recommend that this dissertation prepared under my direction by George Edward Lowke entitled PASSIVE TRANSFER OF HOMOGRAFT SENSITIVITY IN GUINEA PIGS be accepted as fulfilling the dissertation requirement of the degree of DOCTOR OF PHILOSOPHY.

Dissertation Director

Date

After inspection of the final copy of the dissertation, the following members of the Final Examination Committee concur in its approval and recommend its acceptance:*

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SIGNED: George Edward Lerke
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ABSTRACT

The mechanism involved in the rejection of skin homografts in the guinea pig was investigated utilizing passive transfer techniques. Groups of out-bred guinea pigs received orthotopic skin homografts from a donor guinea pig of another strain. These sensitized animals were sacrificed on the 14th day after initial grafting and immune serum, peritoneal exudative cells, regional lymph node cells and alveolar macrophage cells were collected. All three cell populations were consistently effective in passively sensitizing normal animals to the specific skin employed to sensitize the cell donors. Sonic disruption of these cells did not significantly decrease their efficiency in passive transfer of skin graft sensitivity.

Incubation of the sensitive lymphoid cell populations in Hanks' balanced salt solution, normal serum and immune serum caused them to release a protein which was effective in passive transfer. This protein migrated electrophoretically with the serum alpha-1-globulin of guinea pig serum. Although an electrophoretically similar protein was found in incubation fluid of cells from sham-grafted animals, it was present in amounts seven to ten-fold less than in fluid from sensitive animals and was not effective in passive transfer.
Transfer factor was released into normal and immune serum. However, a significant difference was observed in the passive transfer capabilities of these incubation fluids. Immune serum incubation fluid was consistently less effective than normal serum incubation fluid, although only slight differences in the amount of factor released into the two sera could be detected. It was not possible to conclude whether immune serum inhibited the factor after release, or whether it prevented the release of large enough quantities so that the threshold level necessary for passive transfer was not attained.

The results of these experiments support strongly the view that delayed-type hypersensitivity mechanisms are responsible for rejection of homografts. However, the possibility that serum antibody is involved secondarily has not been disproved.
INTRODUCTION

The use of homografts and heterografts in reconstructive surgery and for the replacement of diseased and defective organs has long stimulated the human imagination. Hardly an age has passed without record of some exploratory attempts in this kind of surgery. However, renewed interest in tissue and organ transplantation came not from the clinician, but rather from the research laboratory.

The first systematic attempt at elucidating the mechanism of tissue graft rejection was made by Medawar (1, 2), who observed that when he removed a skin graft from a donor rabbit and placed it on a recipient rabbit, it became fully vascularized and healed into place. After a latent period of 6-8 days, however, the graft began to be rejected by the recipient and became necrotic by the 10th-12th day. If a second graft from the same donor was then placed on the recipient, it was rejected in an accelerated, and much more violent fashion, terminating in rejection of the graft by the 6th or 7th day. This "second-set response" led Medawar to propose that graft rejection is immunological in nature. He suggested that the recipient animal had been stimulated by the first graft so that it demonstrated heightened
sensitivity by rejecting the second graft in an accelerated manner. Temporally, this is similar to the anamnestic response of classical antibody production.

One of the first reports which demonstrated the role of an immune response in bone homotransplantation was that of Bonfiglio, Jeter, and Smith (3). They showed, histologically, that an increased inflammatory response was present in secondary bone grafts and were unable to demonstrate humoral antibodies in the serum of these animals.

The specificity of tissue rejection is another feature which strongly suggests that it is an immunological response. Grafts transplanted to an animal which has previously responded to skin from a different and unrelated donor will usually succumb to a primary, rather than a secondary, response (1, 4). Accelerated rejection of a graft will occur only if the recipient has been exposed previously to tissue from the same donor, or one which is genetically isologous.

There appears to be no doubt that the rejection process is initiated and directed by immunologic mechanisms. The major subject of investigation and debate is the question of whether the vectors of the response are activated migratory leukocytes, humoral antibodies released at a distance from the graft, or some combination of these possibilities.
It is generally recognized that humoral antibody is an almost invariable consequence of the transplantation of skin and other normal tissues, as well as neoplasms. The antibodies thus formed may be detected by hemagglutination (5), leukocyte agglutination (6), hemolysis (7), or cytotoxic reactions (8, 9). There is, however, skepticism regarding the significance of these humoral antibodies in homograft rejection. This is based primarily on repeated failure to achieve passive transfer of homograft sensitivity by large doses of serum in a number of animal species (10, 11, 12, 13, 14, 15, 16, 17, 18) and by the experiments of Algire, Weaver, and Prehn (19) in which homografts enclosed in Millipore chambers, permitting passage of humoral, but not cellular elements, survived for extended periods, even when placed in previously immunized hosts.

There have been reports of successful serum transfer of homograft sensitivity, but they almost invariably involve some artifact such as local injection of antibody around the graft site (20) or attempts to increase the local vascular permeability in the graft by injection of histamine (21). Passive transfer of sensitivity by systemic injection of serum was obtained by Steinmueller (22) who demonstrated a humoral factor in the serum of one isogenic strain of rats (Norwegian brown) which would cause accelerated rejection of a skin homograft from another isogenic strain (Lewis). However, no such factor was
in Lewis rats carrying a Norwegian brown homograft. Also, Norwegian brown rats tolerant of Lewis tissue did not reject Lewis homografts when injected with this serum.

Failure to demonstrate humoral antibody consistently as an effector in homograft rejection has led to much speculation about "cell-bound" antibody (23, 24). This antibody presumably attaches firmly to the lymphoid cells and endows them with the capacity to interact specifically with antigen. Attempts by Warnatz and Scheiffarth (25) to demonstrate specific mouse gamma globulin tightly bound to the surface of cells capable of effecting a specific homograft rejection met with only limited success.

There can be little doubt that leukocytes are intimately involved in homograft rejection. Several investigators have studied the histology of grafts undergoing rejection (2, 3, 4, 26). In first set grafts, infiltrating leukocytes were first seen by the second or third day. They rapidly increased in number and, though finding their way to all parts of the graft, tended to congregate at the graft-host interface. They were present in greatest numbers at the height of rejection. Although the percentage of different types of leukocytes varied at different stages of graft rejection and in different species, the infiltrate was composed of a mixture of cell types, including polymorphonuclear cells, particularly in the early days of graft residence. However, the predominant cells were mononuclear and the commonest cell
approximated a small lymphocyte in appearance (1). The histological appearance of graft rejection, therefore, fulfills one of the major criteria of the delayed hypersensitivity state.

Another major criterion of this state is passive transfer of the sensitivity from sensitive to normal animals with lymphoid cells and not with serum. This was established by Landsteiner and Chase (27) in 1942. Mitchison (12, 14, 15) was the first to meet this requirement in tissue transplantation when he provided evidence to show that immunity to mouse lymphosarcoma was readily transferred to normal mice by means of lymph node cells, but not by means of serum, even when comparatively high titers of antibody could be demonstrated in the donor serum.

Further evidence to support the concept that homograft rejection is a cell-mediated phenomenon has been put forth by several investigators. Billingham, Brent, and Medawar (11) were successful in transferring skin homograft immunity in mice with lymph node cells derived from nodes draining the graft directly. Transfer could not be effected with either lymph node extracts prepared by grinding the cells with sand, freeze-thaw lysis, or lyophilization, or with serum in amounts up to 5 ml. Peritoneal exudative and peripheral blood leukocytes were likewise ineffective. Billingham, Silvers, and Wilson (18, 28) showed that lymph node cells, peripheral leukocytes, and, to a lesser extent, peritoneal exudative cells would passively
sensitize normal mice to skin homografts or would destroy an established graft on a tolerant recipient.

In guinea pigs, Najarian and Feldman (16, 17) transferred lymph node cells from skin sensitized animals to normal recipients and demonstrated immunization against skin grafts. Siebeling (29) was successful in passively sensitizing guinea pigs to skin homografts utilizing lymph node and peritoneal cells, but not serum. Brent, Brown, and Medawar (30, 31) have demonstrated that the reaction against skin homografts can be made to express itself as an apparently typical delayed-type hypersensitivity reaction. When living cells, or cell-free antigenic material, from a skin graft donor were injected intradermally into a sensitized recipient, a "direct reaction," very similar to a tuberculin reaction was provoked. A "transfer reaction" resulted when cells from regional lymph nodes of a sensitized recipient were injected into the original skin donor. This reaction was interpreted as local passive transfer of the reactive state analogous to the local passive transfer of delayed hypersensitivity described by Metaxas and Metaxas-Buhler (32).

Since, in many ways, homograft sensitivity appears to mimic delayed-type hypersensitivity, the present controversy over the possibility of passive transfer of delayed hypersensitivity in animals with fractions of leukocytes cannot be ignored. Crepea and Cooke (33) reported transfer of poison ivy (Rhus radicans) sensitivity in guinea pigs.
with extracts of spleen cells from sensitive animals. Jeter, Tremaine, and Seebohm (34) demonstrated transfer of sensitivity to dinitrochlorobenzene with extracts of peritoneal exudative cells prepared by sonic disintegration. In a later paper, Jeter, Laurence, and Seebohm (35) analyzed extracts of leukocytes from guinea pigs sensitive to tuberculin and dinitrochlorobenzene and reported the presence of a protein, absent from normal cells, which had an electrophoretic mobility similar to serum alpha globulin. In man, Lawrence (36, 37) obtained passive transfer of sensitivity to bacterial proteins with freeze-thaw and distilled water lysates of peripheral leukocytes. He coined the term transfer factor and characterized it as being freely dialyzable, having a molecular weight of 10,000 and not protein in nature. By employing sonically prepared extracts of peritoneal exudative or spleen cells from animals sensitive to tuberculin, Cummings, Patnode, and Hudgins (38) were successful in passive transfer of tuberculin hypersensitivity. Dunn and Patnode (39) later confirmed the findings of Jeter, et al. (35) that leukocytes from sensitive animals contained an abnormally high content of glycoprotein which migrated electrophoretically as an alpha globulin.

In a recent review, Bloom and Chase (40) described attempts to confirm the cell free passive transfer of delayed hypersensitivity as reviewed above, and were unable to do so. However, in view of recent reports by Guthrie, Ellis, and Brock (41), Burger (42, 43) and
by Kucharski and Favour (44), in which passive transfer activity was found in washing fluids and fluids in which sensitive cells were incubated, the possibility exists that activity was lost into the washing fluids before extracts were obtained. This would explain the failure by Chase, and others (31, 45, 46, 47) to obtain passive transfer of sensitivity to chemicals, tuberculin, and homografts with cell free extracts.

Passive transfer of transplantation immunity with subcellular fractions has been achieved by some investigators. Powell, Ray, Whitenack, Hubay, and Holden (48) demonstrated that a skin reaction could be produced in guinea pig skin homograft donors with a cell-free substance extracted from the lymph nodes of animals which had rejected a homograft from the donor. Later, these authors (49) produced accelerated rejection of skin homografts with this cell-free material, which they described as a high molecular weight protein. Accelerated rejection of homografts in mice and guinea pigs following transfer of cells enclosed in millipore chambers was obtained by Najarian and Feldman (16, 17). A similar finding has been reported by Kretschmer and Perez-Tamayo in rabbits (50). Najarian and Feldman subsequently extracted from lymph nodes of skin sensitive mice, a factor which, when given to normal animals, accelerated the rejection of skin homografts (51). Preliminary analysis indicated that
it was a gamma globulin. Thus far, attempts by Billingham, et al. (28) to transfer homograft sensitivity with cell free extracts have failed.

The purpose of this study was to investigate the mechanism of skin homograft rejection in the guinea pig, utilizing passive transfer techniques to test the ability of viable and non-viable leukocytes, incubation fluids, and immune serum to initiate accelerated rejection of skin homografts in previously unsensitized recipients.
CHAPTER 2

MATERIALS AND METHODS

Animals

Outbred albino guinea pigs of the Amana and Rockefeller strains, both male and female, obtained from departmental colonies, were employed throughout this study. The skin donors weighed 800-1000 grams and the skin and passive transfer recipients weighed 500-800 grams. Animals were housed separately under controlled conditions of temperature (25 C) and relative humidity (50%). They were maintained on a diet of Purina guinea pig chow and water containing 0.05% ascorbic acid. Fresh cabbage was given supplementally.

Sensitization Procedure

The skin grafting technique described by Billingham and Medawar (52), as modified by Siebeling (29) was utilized. Skin donor and recipient guinea pigs were anesthetized with sodium nembutal (30 mg/kg) injected intra-abdominally. Ether was used supplementally when required. The abdominal area of the skin donor and the right and left dorso-lateral chest walls of the skin recipients were clipped and shaved free of hair. These surgical areas were then sterilized by scrubbing with 2% Amphil followed by 70% alcohol.
Abdominal skin was removed from the donor in strips approximately 2 x 16 cm and these donor skin deficits were closed with 11 mm Michelle wound clips. The subdermal adipose tissue was then trimmed from the strips and they were cut into individual grafts approximately one cm square. Grafts were stored, raw surface down, on filter paper pads soaked with Hanks' balanced salt solution (HBSS) in petri dishes.

For all experiments, each of sixteen skin recipients received two sensitizing homografts from the donor. The grafts were fitted orthotopically on the dorso-lateral aspect of the rib cage just posterior to the scapulae. Recipient graft beds were prepared by removing a one cm square, full thickness portion of skin. Care was exercised that the panniculus dorsum remained intact so that an ample blood supply could be established to the graft. The graft was held in place with a strip of 3-M Blenderm surgical tape, which was applied directly over the graft and surrounding area. No sutures were employed as the tape provided adequate pressure to prevent slippage and dehydration of the graft. Gauze pads were placed immediately over the taped transplant and the animal was then wrapped with tape to secure and protect the graft site.

Each graft was inspected daily after the fourth post-surgical day to insure that an adequate take was effected and that the normal rejection scheme occurred. The grafts were inspected grossly for
changes in color, integrity of epidermis, and ability to bleed upon being scrapped with a scalpel blade. The tape immediately covering the graft was replaced daily.

**Cell Collection Procedure**

The methods of harvesting peritoneal exudate cells (PEC) and lymph node cells (LNC) were those of Jeter, et al. (34). Forty-eight hours before cell collection 20 ml of sterile light mineral oil were injected intra-abdominally into the skin-sensitized guinea pigs to evoke a mononuclear cell exudate in the peritoneal cavity. Animals were anesthetized with ether or sodium nembutal (when alveolar macrophage cells were collected) and exsanguinated by cardiac puncture. Serum was separated and pooled for injection into recipients or for use as an incubation medium for the cells.

The peritoneal cavity of each animal was opened aseptically and washed three times with heparinized (2 mg/l) HBSS. The washings were collected and centrifuged at 500 x g. for 20 minutes at 25 C. The cell sediments were pooled, washed once and recentrifuged at 500 x g. for 20 minutes. Packed cell volumes were determined and total, viable and differential cell counts were performed.

The lymph nodes which drained the graft site were carefully dissected from the fat pad immediately posterior and ventral to the scapulae. The nodes were minced on a 20 gauge stainless steel screen
into a sterile mortar, the cells expressed from the pulp and suspended in HBSS. They were then centrifuged at 500 x g. for 20 minutes, washed once and recentrifuged. The packed cell volume was recorded and total, viable and differential cell counts were done.

Alveolar macrophage cells (ALC) were collected by alveolar lavage according to the procedure of Myrick, Leak, and Oshima (54). The heart and lungs, with 2-3 cm of trachea, were dissected intact from the pleural cavity. The heart, excess connective tissue and blood clots were then removed from the lungs. Lungs were then washed in two baths of 0.15 M NaCl to remove as much blood as possible. Lavage was performed on lungs from each guinea pig by two washes of 10 ml of HBSS. The cells were pooled, centrifuged, washed once and recentrifuged. Packed cell volumes were determined and total, viable and differential counts were performed.

Cells were collected as quickly as possible, with collections being complete usually within one hour after the first animal was exsanguinated.

**Treatment of Cells**

In all experiments, a portion of the cells (10^9 cells/7.5 ml HBSS) was injected immediately after washing and resuspension into recipient guinea pigs as fresh whole cells. To determine whether non-viable cells retained their capacity to transfer homograft sensitivity,
a gentle method of destroying their integrity was employed to avoid
denaturation of proteins. This involved sonic vibration at 20 kilo-
cycles per second for 5-7 minutes with the cell container immersed in
a water bath at 25 C to prevent excessive heating. When microscopic
examination showed no intact cells remaining, sonication was inter-
rupted and the material was injected into recipients. No attempt was
made to remove the membrane fragments from the soluble material
before injection.

In order to obtain release of the transfer substance into the fluid
in which cells were suspended, in some experiments half of the cells
were incubated in HBSS for four hours at 37 C with frequent gentle
agitation. No attempt was made to control the pH, since it was found
by Burger (43) that there was a better yield of transfer factor from
cells sensitive to dinitrofluorobenzene if the pH was allowed to drop to
6.2 during the incubation period.

After incubation, a portion of the cells was withdrawn for total
and viable counts, and the remainder was centrifuged at 500 x g. for
20 minutes. The supernatant portion was decanted and the cells re-
suspended in HBSS and injected into recipient guinea pigs. The super-
natant fluid was further clarified by centrifugation at 1,200 x g. for
20 minutes. The decanted fluid was then injected into recipient guinea
pigs with a portion being saved for analysis.
To determine the effect of cell incubation in normal and immune serum, a slightly different protocol was followed. Before the animals received sensitizing homografts, 5 ml of blood was withdrawn from each. The serum was collected, pooled and stored at -20°C until the day of passive transfer. On the day of passive transfer, the animals were exsanguinated and the immune serum was collected and pooled. In these experiments, peritoneal and lymph node cells were collected and pooled. After washing and centrifugation, one-fourth of the cells was diluted in HBSS and injected into a recipient as fresh whole cells; one-fourth was diluted in HBSS and incubated four hours at 37°C. One fourth of the cells was diluted to contain $10^9$ cells per 7.5 ml normal serum and incubated and the remaining one-fourth was diluted in the immune serum and incubated. After incubation, the cells and incubation fluids were treated as described above and injected into recipients.

**Passive Transfer Recipients**

Cell, sonicate, incubation fluid and immune serum recipients were of the same strain as the cell donors (skin recipients), and weighed approximately 500 grams. Forty-eight hours after passive transfer, the recipients were challenged by the following procedure. The grafting techniques outlined above were employed except that three grafts were placed one cm apart along the mid-dorsal line of the recipients' backs. These consisted of a challenge graft from the
original skin donor, a control autograft from the recipient's abdomen, and a control homograft from an animal genetically unrelated to the original skin donor. The grafts were inspected daily from the first through the ninth day following placement to determine whether accelerated rejection occurred.

**White Cell Enumeration**

Total and viable white cell counts were made employing standard techniques on the Spencer Bright-Line hemocytometer. Viability was determined by dye-exclusion utilizing Trypan Blue.

To determine the differential count of each cell population, the Janus Green-Neutral Red supravital stain (55) was employed.

**Incubation Fluid Analysis**

A small portion of each of the incubation fluids derived from each experiment was saved for partial analysis. Total protein was determined by the method of Lowry, Rosenbrough, Farr, and Randall (56) utilizing the Folin-Ciocalteau phenol reagent.

Electrophoresis was carried out using the Gelman Rapid Chamber No. 51100 and Sepraphore III cellulose acetate strips.
CHAPTER 3

EXPERIMENTAL RESULTS

In all experiments, the rejection rate of the sensitizing homografts was observed for each animal. Grafts were checked daily from the 5th post-surgical day until they were rejected. On the 5th day they exhibited a pink color which corresponded to that of the surrounding host tissue, and pinpoint bleeding could be demonstrated when the graft was scrapped lightly with a scalpel blade. Grossly, the graft could not be differentiated from an autograft on the 5th day. The same was true on the 6th day. However, a dramatic change was observed during the 7th and 8th days. The color had changed to a brick red to reddish-purple (indicating loss of capillary integrity) and the epidermis was firm to the touch and would not bleed. On the 9th to 10th day the color had transformed to a yellowish-brown and the graft could easily be pulled from the bed.

The mean rejection time was calculated for the 320 animals which were sensitized and was found to be 9.8 days. In no case were the "first-set" grafts rejected before the 8th day and all were rejected by the 12th day. Almost all grafts were rejected on either the 9th or 10th day.
The first set of passive transfer experiments was designed to determine whether homograft sensitivity could be transferred passively to normal recipients by means of leukocytic extracts. Sonic disruption was chosen as a gentle physical means for destroying cellular integrity.

Peritoneal exudative, regional lymph node and alveolar macrophage cells were collected on the fourteenth day after initial grafting. Supravital stained differential counts done on the three cell populations showed that the peritoneal cell population contained 52-59% monocytes, 13-21% large lymphocytes, 19-26% small lymphocytes, 1-5% neutrophils and an occasional eosinophil. The lymph node cell population consisted of 7-19% large lymphocytes, 81-97% small lymphocytes and an occasional neutrophil. Alveolar macrophage populations contained 47-54% monocytes, 40-46% large lymphocytes, 6-11% small lymphocytes and 1-3% neutrophils.

Each cell population was divided into two equal portions. One portion was injected into a recipient as viable whole cells immediately after washing and resuspension. The other portion of each cell population was subjected to sonic vibration until microscopic examination showed no intact cells remaining. This cell-free material was then injected into another recipient for each cell source. All recipients were given a challenge graft 48 hours later. The challenge graft
consisted of skin from the original skin donor. An autograft and an indifferent homograft were also placed on the recipients as controls.

Evidence of successful passive transfer of the sensitivity was manifested in two ways, either as a classical "second-set" rejection, or as a white graft response. In order to be considered a "second-set" response, the challenge grafts had to be rejected by the 6th day. This was considered to indicate a relatively low degree of sensitivity. The white graft response represented an animal so acutely sensitive that no "take" of the graft was ever evident.

Table I shows the effectiveness of passive transfer of whole and sonically disrupted cells in preventing graft take. It can be seen that disrupted peritoneal cells are almost as effective as viable whole cells in passive transfer of homograft sensitivity. The same is true for lymph node cells and for alveolar macrophage cells. However, the recipients of the disrupted cells did not appear to be as highly sensitive as the recipients of whole cells since the number of white grafts was smaller than in the whole cell recipients.

A control experiment was done in which the cell donors were sham-grafted by placing autografts from their abdomens to their post-scapular areas. From this point on, they were treated exactly as the sensitized animals in the above experiments. Cells were collected and injected as whole and sonically disrupted cells. Forty-eight hours later the recipients were challenged utilizing the skin donor of a
TABLE I

PASSIVE TRANSFER OF SKIN GRAFT SENSITIVITY
WITH WHOLE AND SONICALLY DISRUPTED CELLS*

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>No. of Cells x 10^9</th>
<th>Fate of Challenge Grafts</th>
</tr>
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<tbody>
<tr>
<td></td>
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<td>Fate of Graft in Whole Cell Recipient</td>
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<tr>
<td><strong>Peritoneal Exudative Cells</strong></td>
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<td></td>
</tr>
<tr>
<td>1</td>
<td>2.20</td>
<td>White Graft</td>
</tr>
<tr>
<td>2</td>
<td>1.32</td>
<td>White Graft</td>
</tr>
<tr>
<td>3</td>
<td>1.12</td>
<td>Rejected Day 6</td>
</tr>
<tr>
<td>4</td>
<td>1.50</td>
<td>White Graft</td>
</tr>
<tr>
<td>5</td>
<td>1.40</td>
<td>White Graft</td>
</tr>
<tr>
<td><strong>Regional Lymph Node Cells</strong></td>
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<td></td>
</tr>
<tr>
<td>1</td>
<td>0.64</td>
<td>White Graft</td>
</tr>
<tr>
<td>2</td>
<td>0.58</td>
<td>Rejected Day 6</td>
</tr>
<tr>
<td>3</td>
<td>0.83</td>
<td>White Graft</td>
</tr>
<tr>
<td>4</td>
<td>0.99</td>
<td>White Graft</td>
</tr>
<tr>
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<tr>
<td><strong>Alveolar Macrophage Cells</strong></td>
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<tr>
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</table>

* Cells were disrupted at 20 kilocycles per second for 5-7 minutes at 25 C.

** Recipient died before observations completed.
previous experiment. All grafts (except the autografts) exhibited a "first-set" rejection rate.

In view of several reports (41, 42, 43, 44) of successful passive transfer of delayed-type hypersensitivity with fluids derived by incubating sensitive cells in Hanks' balanced salt solution, the second series of experiments was designed to determine whether transfer material responsible for homograft rejection was released into the incubation medium. In each of these experiments, sixteen animals were sensitized to homografts and cells of the three sensitive populations described above were collected on the 14th day after primary grafting. A portion of each cell population was injected as fresh whole cells immediately after washing and resuspension. The other portions were incubated in HBSS for four hours at 37°C. After incubation, the cells were separated from the fluid, and cells and fluids were injected into separate recipients.

Table II presents the data obtained with the peritoneal cell population. Fresh whole cells were effective in five of five attempts with four white graft responses. The cells which had been incubated for four hours were effective in four of five attempts, but with only one white graft response. This lowered degree of sensitivity is not surprising since the pH of the incubation media was allowed to drop to 6.0-6.2 with the resulting loss in viability (from 82-90% to 39-51%). This could mean that the sensitive cells had released their complement
TABLE II

PASSIVE TRANSFER OF SKIN GRAFT SENSITIVITY WITH FRESH WHOLE PERITONEAL CELLS, INCUBATED CELLS AND HBSS INCUBATION FLUID

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>No. of Fresh Whole Cells x 10^9</th>
<th>No. of Cells Incubated x 10^9</th>
<th>Fate of Challenge Grafts</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>1.0</td>
<td>1.8</td>
<td>White Graft</td>
</tr>
<tr>
<td>8</td>
<td>1.0</td>
<td>1.6</td>
<td>White Graft</td>
</tr>
<tr>
<td>9</td>
<td>1.0</td>
<td>2.1</td>
<td>White Graft</td>
</tr>
<tr>
<td>10</td>
<td>1.0</td>
<td>1.9</td>
<td>White Graft</td>
</tr>
<tr>
<td>11</td>
<td>1.0</td>
<td>1.5</td>
<td>Rejected Day 6</td>
</tr>
</tbody>
</table>

*Recipient of cells which were incubated in HBSS for four hours, then separated from the incubation fluid, resuspended and injected.
of transfer factor, then since a portion of them were dead, only the viable cells remaining could produce factor after they were injected into the recipient. The recipients of the incubation fluids exhibited five rejections in five attempts, with three white graft responses, indicating that a relatively high degree of sensitivity was released into the incubation fluid.

The reactivity of recipients receiving fresh and incubated lymph node cells and the fluid derived from incubation is depicted in Table III. As is evident from this table, incubation fluids are quite effective in transferring homograft sensitivity, although perhaps to a lesser extent than fresh whole cells, as are the cells after incubation for four hours. Similar results are shown by Table IV for alveolar macrophage cells. Again the incubation fluid is quite effective in transferring the sensitivity (four of five attempts with two white graft responses). The incubated cells however, appeared to lose almost all of their activity, being effective in only two of five attempts.

If the factor is so readily released in vitro, then it should be released in vivo into the serum. Therefore, passive transfer with immune serum could be obtained. In view of the fact that successful reports of serum transfer are almost nonexistent and reports of unsuccessful attempts are numerous, the following experiments were designed to investigate this point.
### TABLE III

PASSIVE TRANSFER OF SKIN GRAFT SENSITIVITY WITH FRESH WHOLE REGIONAL LYMPH NODE CELLS, INCUBATED CELLS AND HBSS INCUBATION FLUID

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>No. of Fresh Whole Cells $x 10^9$</th>
<th>No. of Cells Incubated $x 10^9$</th>
<th>Fate of Challenge Grafts</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>0.56</td>
<td>0.56</td>
<td>Rejected Day 6</td>
</tr>
<tr>
<td>8</td>
<td>0.91</td>
<td>0.91</td>
<td>White Graft</td>
</tr>
<tr>
<td>9</td>
<td>1.20</td>
<td>1.20</td>
<td>White Graft</td>
</tr>
<tr>
<td>10</td>
<td>0.80</td>
<td>0.80</td>
<td>White Graft</td>
</tr>
<tr>
<td>11</td>
<td>0.69</td>
<td>0.69</td>
<td>Rejected Day 6</td>
</tr>
</tbody>
</table>

*Recipient of cells which were incubated in HBSS for four hours, then separated from the incubation fluid, resuspended and injected.

**Recipient died before observations completed.
TABLE IV
PASSIVE TRANSFER OF SKIN GRAFT SENSITIVITY WITH FRESH WHOLE ALVEOLAR MACROPHAGE CELLS, INCUBATED CELLS AND HBSS INCUBATION FLUID

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>No. of Fresh Whole Cells x 10⁹</th>
<th>No. of Cells Incubated x 10⁹</th>
<th>Fate of Challenge Grafts</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>0.46</td>
<td>0.46</td>
<td>White Graft</td>
</tr>
<tr>
<td>8</td>
<td>0.51</td>
<td>0.51</td>
<td>White Graft</td>
</tr>
<tr>
<td>9</td>
<td>0.39</td>
<td>0.39</td>
<td>Rejected Day 6</td>
</tr>
<tr>
<td>10</td>
<td>0.42</td>
<td>0.42</td>
<td>Rejected Day 6</td>
</tr>
<tr>
<td>11</td>
<td>0.50</td>
<td>0.50</td>
<td>White Graft</td>
</tr>
</tbody>
</table>

*Recipient of cells which were incubated in HBSS for four hours, then separated from the incubation fluid, resuspended and injected.
Animals were sensitized to homografts and peritoneal cells, lymph node cells and immune serum were collected at 14 days after grafting. Normal serum was collected before sensitization was initiated. Peritoneal and lymph node cells were pooled and the resulting cell suspension was divided into four equal portions. One portion was injected as fresh whole cells; one portion was incubated in HBSS; one portion was incubated in normal serum; and the remaining portion was incubated in immune serum. After incubation, the cells were separated from their respective incubation fluids and cells and incubation fluids were injected into separate recipients.

Table V shows the effectiveness of the cells, both fresh and incubated, in passive transfer of homograft sensitivity. As expected, the fresh whole cells were quite efficient (nine of nine attempts with six white graft responses). Cells incubated in HBSS exhibited a lowered degree of sensitivity as before, with seven successes in nine attempts and no white grafts. A slight difference can be seen between the recipients of cells incubated in normal and immune sera. There were nine successful transfers in nine attempts with six white graft responses when cells were incubated in normal serum as opposed to seven of nine with only two white grafts when cells were incubated in immune serum.

An even more significant difference between normal and immune serum incubation can be seen in Table VI, which shows the
### TABLE V

PASSIVE TRANSFER OF SKIN GRAFT SENSITIVITY WITH FRESH WHOLE CELLS* AND CELLS INCUBATED IN HBSS, NORMAL SERUM AND IMMUNE SERUM

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>No. of Cells Injected x $10^9$</th>
<th>Recip. of Fresh Whole Cells</th>
<th>Recip. of Cells Incubated in HBSS</th>
<th>Recip. of Cells Incubated in Normal Serum</th>
<th>Recip. of Cells Incubated in Immune Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>1.13</td>
<td>White Graft</td>
<td>Rejected Day 6</td>
<td>White Graft</td>
<td>Rejected Day 6</td>
</tr>
<tr>
<td>13</td>
<td>0.96</td>
<td>Rejected Day 6</td>
<td>Survival</td>
<td>Rejected Day 6</td>
<td>Survival</td>
</tr>
<tr>
<td>14</td>
<td>1.22</td>
<td>White Graft</td>
<td>Rejected Day 6</td>
<td>White Graft</td>
<td>Rejected Day 6</td>
</tr>
<tr>
<td>15</td>
<td>1.40</td>
<td>White Graft</td>
<td>Rejected Day 6</td>
<td>White Graft</td>
<td>Rejected Day 6</td>
</tr>
<tr>
<td>16</td>
<td>1.02</td>
<td>Rejected Day 6</td>
<td>Rejected Day 6</td>
<td>Rejected Day 6</td>
<td>Rejected Day 6</td>
</tr>
<tr>
<td>17</td>
<td>1.31</td>
<td>White Graft</td>
<td>Rejected Day 6</td>
<td>White Graft</td>
<td>White Graft</td>
</tr>
<tr>
<td>18</td>
<td>0.89</td>
<td>Rejected Day 6</td>
<td>Survival</td>
<td>Rejected Day 6</td>
<td>Survival</td>
</tr>
<tr>
<td>19</td>
<td>1.15</td>
<td>White Graft</td>
<td>Rejected Day 6</td>
<td>White Graft</td>
<td>Rejected Day 6</td>
</tr>
<tr>
<td>20</td>
<td>1.42</td>
<td>White Graft</td>
<td>Rejected Day 6</td>
<td>White Graft</td>
<td>White Graft</td>
</tr>
</tbody>
</table>

*Peritoneal exudative and lymph node cells pooled.

**Number represents one-fourth of total cell population, viz. each recipient received one-fourth of the cell population.
TABLE VI

PASSIVE TRANSFER OF SKIN GRAFT SENSITIVITY WITH HBSS, NORMAL AND IMMUNE SERUM INCUBATION FLUIDS AND IMMUNE SERUM *

Fate of Challenge Grafts

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>HBSS Fluid Recipient</th>
<th>Normal Serum Fluid Recip.</th>
<th>Immune Serum Fluid Recip.</th>
<th>Immune Serum* Recipient</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>Rejected Day 6</td>
<td>Rejected Day 6</td>
<td>Survival</td>
<td>Survival</td>
</tr>
<tr>
<td>13</td>
<td>Rejected Day 6</td>
<td>Rejected Day 6</td>
<td>Survival</td>
<td>Survival</td>
</tr>
<tr>
<td>14</td>
<td>Rejected Day 6</td>
<td>Rejected Day 6</td>
<td>Survival</td>
<td>Survival</td>
</tr>
<tr>
<td>15</td>
<td>White Graft</td>
<td>d**</td>
<td>Rejected Day 6</td>
<td>Rejected Day 6</td>
</tr>
<tr>
<td>16</td>
<td>Rejected Day 6</td>
<td>Rejected Day 6</td>
<td>Survival</td>
<td>Survival</td>
</tr>
<tr>
<td>17</td>
<td>White Graft</td>
<td>Rejected Day 6</td>
<td>Survival</td>
<td>Survival</td>
</tr>
<tr>
<td>18</td>
<td>Survival</td>
<td>Survival</td>
<td>Survival</td>
<td>Survival</td>
</tr>
<tr>
<td>19</td>
<td>Rejected Day 6</td>
<td>Rejected Day 6</td>
<td>Survival</td>
<td>Survival</td>
</tr>
<tr>
<td>20</td>
<td>White Graft</td>
<td>White Graft</td>
<td>Rejected Day 6</td>
<td>Survival</td>
</tr>
</tbody>
</table>

*Ten cc of immune serum injected as a control.

**Recipient died before observations completed.
results obtained when passive transfers with incubation fluids were attempted. Normal serum incubation fluids were effective in seven of eight attempts with one white graft response, whereas immune serum incubation fluid was successful in only two of nine attempts with no white graft responses. Thus it appears that immune serum somehow prevents the expression of the transfer material, either by preventing its release from the sensitive cells, or perhaps by inactivating it as it is released. It should be noted, however, that one of the nine immune serum control recipients exhibited accelerated rejection of the challenge graft.

A similar experiment was conducted as a control in which animals were sham-grafted with autografts, their cells collected at fourteen days and treated as above, i.e., incubated in HBSS, normal serum and "immune" serum. The recipients were guinea pigs of the same strain as the cell donors and challenge grafts were taken from the skin donor of a previous experiment. All challenge grafts were rejected in a "first-set" fashion.

It is noteworthy that in some cases, the indifferent homografts placed on passive transfer recipients as a control were rejected in an accelerated fashion, but rarely as a white graft. This could possibly indicate that the phenomenon of passive transfer of homograft sensitivity utilizing the systems employed in this study is non-specific immunologically. Absolutely no pattern could be determined, i.e., no
one type of recipient consistently exhibited this response. Therefore, the occasional accelerated response to indifferent homograft controls is interpreted as indicating that the indifferent skin donor (from the same strain, but presumably genetically unrelated to the sensitizing skin donor) nevertheless shared histocompatibility antigens, in varying degrees, with the skin donor. It could also possibly indicate a slight degree of technical failure on the part of the surgeon in placing the grafts. However, no significance is attributed to this possibility since 100% of the autograft controls placed on the recipients survived.

A preliminary analysis of the incubation fluids was carried out. This consisted of determination of protein concentration and electrophoretic analysis. The protein concentration of HBSS incubation fluids in experiments 7-11 was found to range from a high of 31.5 mg to a low of 11.5 mg, depending on the population of cells incubated. In general, the peritoneal cells released the largest amounts of protein, followed by alveolar cells and lymph node cells in that order. The variance in amounts of factor released could possibly be due to the variance of cell types found in each cell population.

Table VII shows the protein concentrations of incubation fluids derived by incubating pooled peritoneal exudative and lymph node cells in HBSS, normal serum and immune serum. It can be seen that, for any given experiment, more protein was released into HBSS than into normal or immune serum. Also, normal serum appeared to contain
TABLE VII

PROTEIN CONCENTRATION* OF HBSS, NORMAL SERUM AND IMMUNE SERUM INCUBATION FLUIDS**

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>2.70</td>
<td>2.20</td>
<td>1.60</td>
</tr>
<tr>
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<td>3.60</td>
<td>2.90</td>
<td>2.30</td>
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<td>3.40</td>
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<td>2.60</td>
</tr>
<tr>
<td>18</td>
<td>1.20</td>
<td>0.95</td>
<td>0.80</td>
</tr>
<tr>
<td>19</td>
<td>3.10</td>
<td>2.70</td>
<td>2.30</td>
</tr>
<tr>
<td>20</td>
<td>3.65</td>
<td>3.10</td>
<td>2.70</td>
</tr>
<tr>
<td>21***</td>
<td>0.45</td>
<td>0.30</td>
<td>0.27</td>
</tr>
</tbody>
</table>

*All protein concentrations are given as mg/ml. Cells for incubation were diluted in the ratio of 10^6 cells/7.5 ml of medium (HBSS, normal serum, or immune serum).

**The protein concentration shown for the serum incubation fluids represents the amount of protein released by the cells, i.e., the protein concentration of the serum incubation fluids minus the protein concentration of serum from the same source which had not been incubated with cells.

***These figures represent the protein released from presumably non-sensitive cells, i.e., cells collected from animals which had been sham-grafted.
more protein after incubation with cells than the immune serum. It appears therefore, that immune serum inhibited, to a degree, the release of transfer factor. However, the difference in protein concentration between normal and immune serum incubation fluids are not large enough to be completely out of the range of experimental error. Although the trend of less protein released into immune than normal serum appeared to be consistent, the differences between their passive transfer capabilities, seen in Table VI, cannot necessarily be attributed to the slight differences in protein concentration. Since dosage response studies were not undertaken in these experiments, it is impossible to determine if the amount of factor released into the immune serum incubation fluids was below the threshold level necessary to effect passive transfer of homograft sensitivity.

The average percent cellular viability for these series of experiments was: incubation in HBSS, 49%; incubation in normal serum, 67%; and incubation in immune serum, 42%. The pH of HBSS incubation fluid was 6.1-6.2. There was however, no significant difference between the pH of normal and immune serum incubation fluids (6.9-7.1).

Electrophoretic analysis of HBSS incubation fluids showed a single band which migrated similarly to serum alpha-1-globulin. The protein released into normal and immune serum upon incubation migrated with the serum alpha-1-globulin. The increased concentration
in the alpha-1-band (in the serum incubation fluids as opposed to serum from the same source which had not been incubated with cells) could be calculated to account for essentially all of the protein released from the cells. This does not necessarily indicate that the transfer factor released is an alpha-1-globulin, only that it possesses electrophoretic characteristics similar to serum alpha-1-globulin.
CHAPTER 4

DISCUSSION

The mechanism underlying the rejection of homografts is incompletely understood. There appears to be no doubt that the process is set in motion and directed by immunologic mechanisms. Although some evidence is available which indicates that responsibility for graft damage can be ascribed to serologic antibody, the overwhelming majority of reports in the literature favors a mode of reaction mediated in some way by sensitive leukocytes. Therefore, it is probably similar to a delayed-type hypersensitivity phenomenon.

The mechanism involved in delayed-type hypersensitivity has been studied a great deal, but also remains unresolved. That sensitive cells mediate this response is an established fact. Precisely how these cells are involved is the subject of much investigation and debate. Some authors (40, 46) steadfastly maintain that passive transfer of the sensitivity demands viable cells. However, there is mounting evidence to show that passive transfer is readily achieved by subcellular fractions of leukocytes. The first successful attempts at passive transfer with such fractions of leukocytes sensitive to chemicals were reported by Jeter, et al. (34, 35). An analysis of the extracts obtained revealed a
fraction, absent from normal cells, with an electrophoretic mobility analogous to serum alpha globulin.

Confirmation of the subcellular passive transfer in animals has been reported from several laboratories (38, 39, 41, 57, 58), utilizing the chemical system as well as bacterial hypersensitivities. Lawrence (36, 37) has reported similar findings in man, but characterized his transfer factor as being of low molecular weight, dialysable, and not protein in nature.

Due to the many similarities between delayed-type hypersensitivity and homograft sensitivity, the work reported here was initiated on the premise that a delayed-type hypersensitivity mechanism was involved. The initial experiments were designed to investigate the possibility of passive transfer of homograft sensitivity in guinea pigs by means of sonically disrupted leukocytes. Three leukocytic cell populations were collected from animals sensitive to homografts. One-half of the cells was disintegrated by sonic vibration, then injected, and half was injected as whole viable cells. The results obtained showed that sonically disrupted cells were almost as effective in passive transfer as whole cells. This does not agree with the work of Billingham, Brent, and Medawar (11) who were unsuccessful in attempts to transfer skin sensitivity in mice with extracts of lymph node cells prepared by grinding the cells with sand, freeze-thaw lysis, or lyophilization. The experiments reported here do agree, in principle at
least, with those of Powell, et al. (49) who succeeded in transferring skin sensitivity in guinea pigs with a "partially purified transfer factor," extracted from homogenates of lymph node cells, which they described as being a high molecular weight protein. Najarian and Feldman (51) have likewise been successful utilizing disrupted leukocytes. They reported passive transfer of skin sensitivity in mice with a "soluble substance," extracted from sonically disrupted lymph node cells, which had physiochemical characteristics similar to gamma globulin. Lawrence, Rapaport, Converse, and Tillett (59) have reported both local and systemic transfer of homograft sensitivity in man utilizing desoxyribonuclease-treated leukocyte extracts.

In view of several recent reports (41, 43, 44) which indicated that a protein responsible for passive transfer of delayed hypersensitivity was released by sensitive cells into washing fluids and fluids in which the cells were incubated, the second group of experiments was designed. In these experiments, the three sensitive cell populations were incubated in HBSS. Incubation fluids obtained by this technique were shown to be both effective and specific in passive transfer of homograft sensitivity. This phenomenon has not been reported before in the literature. The only previous report of such an effect in passive transfer of homograft sensitivity was that of Hinrichs (60) in doubly sensitized animals.

Analysis of the incubation fluids derived from both sensitive cells and cells from autografted animals showed that protein was
present in both which had electrophoretic characteristics similar to serum alpha-1-globulin. However, the sensitive cells released seven to ten-fold more protein than cells from the sham-rafted animals, and only fluids derived from sensitive animals were effective in passive transfer.

The literature contains many reports of unsuccessful attempts at serum transfer with only an occasional report of passive transfer with immune serum. Since a factor responsible for passive transfer of homograft sensitivity is readily released in vitro, one wonders if it could possibly be released in vivo, and if so, why passive transfer apparently cannot be effected with immune serum. The third group of experiments was designed in an attempt to resolve this question.

Sensitive cells were collected as before and incubated in normal and immune serum as well as HBSS. Passive transfer with the incubation fluids showed a significant difference between those derived from normal serum and those derived from immune serum. Incubation in immune serum appeared to suppress the expression of the transfer factor, either by preventing its release from the cells or by inhibiting its activity after it was released. There was 15-28% less protein released into immune serum than into normal serum. This would indicate that perhaps the first possibility is true. However, if this was the case, the cells incubated in immune serum should retain most of their passive transfer capability. This was not true as the
cells incubated in immune serum showed considerably less sensitivity on passive transfer than did the cells incubated in normal serum. Even normal serum appeared, to a certain extent, to inhibit the release of transfer factor, as there was 10-22% less protein released into normal serum than into HBSS. Another point to consider is the fact that the average percent viability after incubation in immune serum was less (42%) than in normal serum (67%). This could mean that possibly a cytotoxic antibody was present which simply killed a portion of the sensitive cells, thereby inhibiting both the release of the factor and its subsequent production by the cell. Cytotoxic antibodies have been shown to be present in serum of animals which have rejected homografts (8, 9). One might question the possibility of a cytotoxic antibody being effective against cells from the same strain of guinea pigs since immune serum and cells were collected from the same animals in each experiment. However, the animals used in these experiments were not inbred animals and it is possible that a portion of the cells shared, in varying degrees, histocompatibility antigens with the skin donor. Since the percentage of viable cells was approximately the same when cells were incubated in immune serum or HBSS and the cells incubated in immune serum were effective in passive transfer, a strong case for the presence of cytotoxic antibody cannot be made.
The possibility of inhibition of the factor after release by immune serum has neither been proved nor disproved by these experiments. An experiment which might indicate this possibility would be to obtain release of the factor in HBSS, concentrate it, then incubate it with immune serum and attempt passive transfer. However, due to the limitations of time and animals, this experiment was not done.

It is interesting that one of the nine immune serum control recipients exhibited an accelerated rejection of the challenge graft. This could possibly indicate that a humoral factor (either the transfer released in vivo, or a classical antibody) was present in the immune serum which caused graft rejection. There are a few reports in the literature which indicate that humoral antibody may play a part in graft rejection although they do not involve the critical technique of passive transfer. Gamma globulin which bound complement was shown to be present in the walls of afferent glomerular arterioles of transplanted kidneys undergoing rejection in dogs by Horowitz, Burrows, Paronetto, Drelling, and Kark (61). A similar finding has been reported by Porter (62) in human renal homografts undergoing late rejection after immunosuppressive therapy. Working with delayed hypersensitivity systems, Asherton and Loewi (63) have shown that immune serum produces a synergic effect on passive transfer of sensitivity to protein antigens. A recent paper by Dupuy, Perey, and Good (64) claimed successful passive transfer of tuberculin sensitivity
in guinea pigs with plasma from animals which had been sensitized, then X-irradiated. These papers indicate the possibility of a humoral factor being involved in delayed-type hypersensitivity.

Care must be taken when discussing the participation of delayed hypersensitivity in transplantation immunity that the possible involvement of humoral factors is not forgotten. Although the results discussed here tend to support strongly the view that delayed-type hypersensitivity mechanisms play the primary role in rejection of homografts, the possibility that serum antibody is involved secondarily has not been disproved.
CHAPTER 5

SUMMARY

The mechanism underlying the rejection of skin homografts in guinea pigs was investigated utilizing passive transfer techniques. Peritoneal exudative, lymph node and alveolar macrophage cells, collected from animals which had rejected a skin homograft, were consistently effective in passively sensitizing normal animals to the specific skin employed to sensitize the cell donors. These cell populations were also quite efficient in initiating a state of reactivity to the skin grafts when they were sonically disrupted before passive transfer was attempted.

In view of several reports in which a factor responsible for passive transfer of delayed-type hypersensitivity to chemicals was found in fluids in which sensitive cells were incubated, the second series of experiments was designed. The above three sensitive cell populations were incubated in Hanks' balanced salt solution for four hours. The incubation fluids derived from these experiments readily transferred homograft sensitivity to normal recipients. Preliminary analysis carried out on these fluids indicated that 11.5 to 31.5 mg of protein was released by the cells during incubation. This protein
migrated electrophoretically similarly to serum alpha-1-globulin.
Although an electrophoretically similar protein was found in incubation fluid of cells from sham-grafted animals, it was present in amounts seven to ten-fold less than in fluid from sensitive animals, and was not effective in passive transfer.

Since a factor responsible for passive transfer of homograft sensitivity is readily released in vitro from sensitive cells into a minimal incubation medium, the question of whether the cells release the factor in vivo arises and if so, whether the serum then inhibits the factor so that passive transfer with immune serum cannot be effected. This point was investigated by incubating cells sensitive to homografts in normal serum and immune serum, then testing both the cells and serum incubation fluids for their ability to transfer the sensitivity to normal recipients. Only a slight difference could be noted between cells incubated in normal serum and those incubated in immune serum. However, a significant difference was seen between normal serum incubation fluids and immune serum incubation fluids. Normal serum incubation fluids transferred the sensitivity nine times in nine attempts with a relatively high degree of sensitivity, whereas immune serum incubation fluids were effective only twice in nine attempts with a low degree of sensitivity.

This indicated that the expression of the transfer factor was being suppressed. Preliminary analysis of the serum incubation fluids
indicated that less protein was released into immune serum than into normal serum. However, due to the amount of protein released into immune serum, it is impossible to conclude whether immune serum inhibits the factor after release, or simply prevents its release by the cells in quantities large enough to keep that which is released below the threshold level necessary to effect passive transfer.
LITERATURE CITED


