PASSIVE TRANSFER OF TRANSPLANTATION IMMUNITY*‡

I. Tritiated Lymphoid Cells

II. Lymphoid Cells in Millipore Chambers

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Immunologic mechanisms involved in homograft rejection and tuberculin sensitivity have been suggested to be similar modalities of delayed sensitivity (1). Cogent evidence for this proposed similarity has been the consistent finding of the essentiality of the lymphoid cell in the passive transfer of both of these reactions (2). In tuberculin sensitivity, significant numbers of sensitized lymphoid cells have been shown to accumulate at the site of the tuberculin skin test following passive intravenous transfer of labeled sensitized cells. Sensitized lymphoid cells did not accumulate at the injection site of non-specific antigen nor were non-sensitized cells attracted to the site of PPD application (3). In addition, BCG-sensitized cells enclosed in millipore chambers were found to be ineffective in the transfer of tuberculin sensitivity (4).

This study was designed to examine the skin homograft site after cells, labeled with tritiated thymidine and sensitized to the homologous skin donor, were passively transferred to homografted isologous hosts. With this technique, the number, types, and distribution of sensitized cells could be ascertained in the rejection site. In addition, the ability of sensitized cells to destroy a homograft at a distance was studied by transferring transplantation immunity with sensitized cells enclosed in millipore chambers impermeable to the passage of cells.

Materials and Methods

I. Tritiated Lymphoid Cells.—

Two inbred strains of mice, A and CBA,[1] were used throughout the study. All skin grafts were prepared as full thickness grafts. The panniculus adiposus and panniculus carnosus were

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[1] 2 C3H mice were used as donors of skin for some controls in the millipore chamber transfers.

removed from the under surface of the dermis by sharp dissection. The grafts were fitted orthotopically into recipient beds and held in place with either interrupted or continuous 5-0 silk sutures. The sensitizing skin grafts were cut into equilateral triangles measuring 2 cm on each side and placed on recipients with the apex of the graft in the nuchal region and the base crossing the scapulae. This arrangement permitted harvesting of sensitized cells from regional cervical and axillary lymph nodes of prospective donor mice. The test skin grafts were cut into rectangles measuring 1 by 2 cm and were sutured into defects over the lumbar area of the back, thus permitting removal of both the graft and the graft bed at the time of biopsy. Both the sensitizing grafts and the test grafts were handled under sterile conditions and were not covered by dressing after placement. The recipient mice were isolated in individual cages and were thus prevented from disturbing each other's grafts, and by virtue of the position of the grafts from disturbing their own.

Donor mice of each strain were sensitized to the homologous strain by cross-skin grafts and intraperitoneal injection of 10^7 spleen cells, two days after grafting. The donor mice were injected intraperitoneally with 0.25 μc of tritiated thymidine per gm of body weight every 8 hours for 3 days prior to cell transfer. 8 hours after the last isotope injection, 9 days after

TABLE I

The Differential Counts and Per Cent of Labelled Cells in the Cell Transfer Suspensions

Mononuclear cell type	Per cent of total Cells*	Per cent cell type labeled	Per cent cell type of total labeled cells
Small, $<8\mu$	48	24	36
Medium, 8 to 12 μ	42	39	48
Large, $> 12\ \mu$	9	58	16

* The remaining 1 per cent includes polymorphonuclear leukocytes, megakaryocytes, and other unidentified cell types.

grafting, the cervical and axillary lymph nodes and spleens were removed and shredded with rakes in balanced salt solution (Hanks') containing 20 per cent PVP by volume. The resulting suspension was passed through an 80 gauge monel gauze filter. Smears were made for differential analysis and autoradiography. Total numbers were determined in a hemocytometer, and cell viability was measured by staining with 1:1000 trypan blue.

An average of 33 per cent of the cells in suspension were labeled. In Table I are shown the differential counts of the mononuclear cells, the percentage of each cell type labeled, and the proportion of each cell type labeled within the total number of tritiated elements. Over 90 per cent of the transferred cells were viable as indicated by the trypan blue staining technique.

The lymphoid cell suspensions were injected into the recipient mice in doses ranging from two to six lymphoid equivalents.[2] Transfer of cells was achieved by intravenous or a combination of intravenous and intraperitoneal administration to A mice and by intraperitoneal administration to CBA mice. When the intravenous route was used only 2 lymphoid equivalents were given since higher doses resulted in prohibitively elevated mortality rates in the recipients. When the intraperitoneal route was used the residual cellular brei was included in the transfer along with the cell suspension.

To investigate different combinations of duration of cell transfer and duration of skin

[2] A lymphoid equivalent is the number of regional lymph node and spleen cells obtained from a single donor mouse, and in this experiment ranged between 160 and 240 $\times$ 10^6 lymphoid cells.

grafting, test skin homografts were placed on isologous recipients from 3 days before to 3 days after the transfer of sensitized cells. The recipient mice were then sacrificed at daily intervals up to 6 days after skin grafting. The test graft sites and also the spleens, lymph nodes, and other pertinent organs were processed for histology and autoradiography.

In a series of 6 animals, sensitized labeled lymph nodes removed 9 days after skin grafting were sliced into small fragments and placed subcutaneously around skin homografts on recipient isologous mice. Skin graft sites, lymph nodes, and spleens were removed from these animals at daily intervals up to 6 days for histology and autoradiography.

For histology, tissues were stained with hematoxylin and eosin. For autoradiography, the unstained tissues and smears were coated with liquid Kodak NTB-2 or NTB-3 track emulsions. After 18, 30, and 60 day exposure periods, the autoradiographs were developed and tissue sections were stained with hematoxylin and smears with Azure B bromide.

II. Lymphoid Cells in Millipore Chambers.—

Suspensions of sensitized lymph node and spleen cells, without isotopic labeling, were prepared in the same manner from donor A and CBA mice as described above. The suspensions, including tissue brei, were placed in millipore chambers 13 mm in diameter with VC[3] membranes having a pore size of 0.1μ and a thickness of 150μ. These chambers were constructed by attaching the VC membrane to a lucite ring (13 mm in diameter, 2 mm wide and 2 mm thick) using MF[3] cement, formulation No. 1, to obtain a quick-drying bond between the membrane and the lucite ring. The lucite ring and attached membrane were sterilized in 1:750 aqueous zephiran chloride[4] and rinsed in sterile saline. Each chamber was found to hold approximately one lymphoid equivalent in an 0.1 ml volume. After the cell suspension was placed in the chamber with a sterile syringe, the chamber was sealed by attaching a second 13 mm VC filter to the open end of the lucite ring with MF cement. The filled chambers were immersed in balanced salt solution containing 20 per cent PVP by volume and 100,000 units aqueous penicillin per ml in preparation for transfer to recipient mice. The time lapse from removal of the lymph nodes and spleens to insertion of the millipore chambers never exceeded 90 minutes.

The recipient isologous mice were divided into two experimental groups. In the first group of 30 mice, two or three millipore chambers containing sensitized cells were placed in the peritoneal cavity. Homologous skin grafts were applied to the dorsa of these recipients two days later. In the second group of 36 animals, three or four millipore chambers were placed subcutaneously around the periphery of the graft bed and the test homograft was sutured into place. The skin grafts from both groups were biopsied at 3 and 6 days after grafting and sections were taken for histology.

For controls, millipore chambers containing non-sensitized isologous lymphoid cells were placed in the peritoneal space of 16 mice and subcutaneously around homografts of skin in 26 mice. The mice with intraperitoneal chambers with skin homografted 2 days later. Biopsies were obtained for histology from both groups 6 days after skin grafting.

In 6 CBA mice with intraperitoneal millipore chambers containing cells sensitized to A tissues a C3H skin graft was applied in addition to the A skin graft. This control was used to determine the specificity of the effect observed on skin homografts by cells sensitized to the homologous donor and enclosed in millipore chambers. C3H mice were chosen as the third strain since they share the same allele at the H-2 locus ($H\text{-}2^k$) as the CBA host and differ at the H-2 locus from the donor A skin homograft ($H\text{-}2^a$).

In a final group "immune" serum[5] was obtained from 66 CBA and A mice on the 9th day

[3] Obtained from Millipore Filter Corporation, Bedford, Massachusetts.

[4] Obtained from Winthrop Laboratories, New York.

[5] An average of 0.33 ml of serum obtained from each sensitized mouse.

after grafting by terminal bleeding of the donors for the millipore experiments. The serum was then separated from the whole blood and in 4 animals (2 A and 2 CBA) 1.67 ml of this serum was administered intraperitoneally 2 days after skin grafting. In addition, another group of 4 mice was given daily intraperitoneal injections of 0.67 ml of immune serum from the day of grafting to biopsy on the 6th day after grafting; thus each one received a total of 4.0 ml.

The test skin homografts on all recipient mice were scored by gross and microscopic examination. When the gross and microscopic score did not correspond (15 per cent of readings), the microscopic appearance determined the final category of the graft site. Tests grafts were classified in one of three categories: (*a*) complete rejection or more than 90 per cent destruction of the transplanted skin; (*b*) no rejection or less than 10 per cent necrosis of the test graft; and (*c*) partial rejection or necrosis, which included from 10 per cent to 90 per cent necrosis of the homograft. The test skin homografts were removed with 6 days of placement. This time interval was chosen since it has been shown that with exchange skin grafts between A and CBA mice the MST[6] of second set grafts is 6 days and that of first set skin grafts is 10 to 11 days (5). Therefore, all grafts which showed partial or complete rejection within 6 days after grafting were classified as accelerated rejections.

TABLE II

Passive Transfer of Labeled Cells Sensitized to Skin Grafts

Mice		No rejection	Partial rejection or necrosis	Complete rejection	No. of graft sites with labeled cells
Strain	Number				
A	21	6	11	4	1
CBA	25	1	12	12	8
Totals.....	46	7	23	16	9*

* Represents a total of 27 labeled cells found in 412 autoradiographs.

RESULTS

I. Tritiated Lymphoid Cells.—The transfer of sensitized labeled lymphoid cells to isologous hosts resulted in macroscopic and microscopic evidence of accelerated homograft rejection in 39 of 46 recipient mice (Table II). 16 of these were complete rejections and 23 exhibited varying degrees of destruction. Despite effective passive transfer of homograft immunity and a 33 per cent label of the transferred cells, only nine graft sites[7] disclosed the presence of a total of 27 labeled cells in 412 autoradiographs.

The length of time the graft was in place, the length of time the transferred cells were in the recipient, the length of time that transferred cells and test homografts were present together in the host, the route and number of cells transferred, were variables that did not affect the small number of labeled sensitized cells that was found in the test graft site (Fig. 1).

[6] Median survival time.

[7] Skin graft biopsy sites included donor skin and host bed.

The effectiveness of passive transfer of transplantation immunity, however, was influenced by some of the technical manipulations listed above (Fig. 1). It was observed that the homograft usually had to be in residence for 2 days before rejection could be demonstrated. 5 of the 7 grafts in Table II showing no rejection were biopsied within 24 hours after grafting and the remaining two grafts were biopsied within 48 hours after grafting. Although 2 days were required for the onset of rejection, complete graft rejection was evident in 10 of 19 grafts bi-

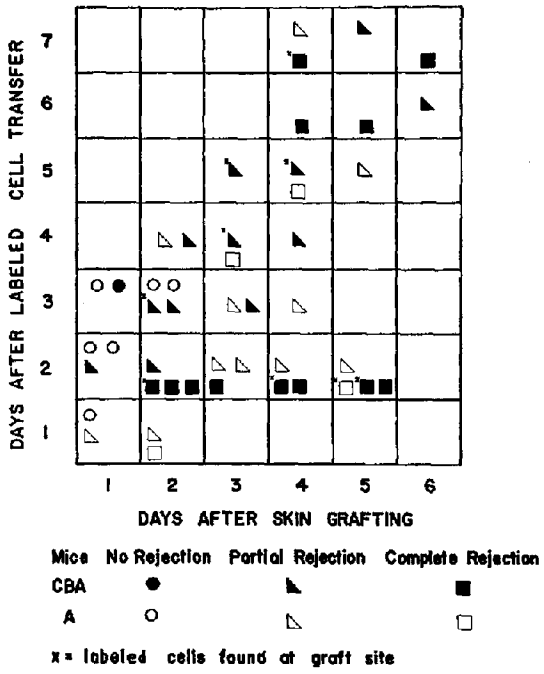

FIG. 1. Relationship of time of biopsy to transfer of labeled cells and skin homograft following passive transfer of transplantation immunity.

opsied after 72 hours residence and the remaining nine showed greater than 50 per cent destruction. Route of administration of sensitized cells also influenced the time of onset of rejection. When the cells were given intravenously, beginning rejection could be seen in 2-day-old grafts as early as 24 hours after cell transfer. However, when the intraperitoneal route was employed (all CBA mice), the earliest signs of rejection did not appear until 48 hours after cell transfer. Finally, it was learned that at least two lymphoid equivalents were necessary to transfer transplantation immunity in this system. The effect of increasing doses up to six lymphoid equivalents (1.2×10^9 cells) gave a more accelerated response at the expense of a higher mortality rate and no appreciable quantitative or qualitative change in the morphology of the rejection siste.

The labeled cells appeared to be mature, small- and medium-sized lymphocytes. 26 of the 27 cells were present in the host's graft bed and the remaining labeled cell was present in the graft itself. 18 of the 27 labeled cells were found in 2 animals, 10 in an A mouse, and 8 in a CBA mouse. However, there was no common denominator between these two chance findings since the route of injection and the relationship of cell transfer to graft biopsy were different in the 2 recipients.

The success of labeling and transferring the cells was measured by the ease of finding labeled cells in the lymph nodes and spleens of the recipients for as long as 7 days after cell transfer. Other non-lymphoid parenchymal organs rarely showed a labeled cell 24 hours after cell transfer. In one animal which died 4 minutes after intravenous injection of labeled cells, the tritiated cells could be found abundantly in the lung and liver, and to a lesser degree, in the kidney, but not in the spleen and lymph nodes.

In the 6 mice in which labeled lymph node fragments were placed subcutaneously around the skin homografts, there was partial rejection of the grafts within 2 days. However, the process of rejection remained partial and was not complete even at the 6th post-graft day. The areas of partial rejection usually corresponded to the areas of the graft in proximity to the sensitized lymph node fragments. Labeled cells could be identified readily in the lymph node fragments but were not present in any of these graft rejection sites.

II. Lymphoid Cells in Millipore Chambers.—Passive transfer of transplantation immunity was achieved in a significant number of recipient mice which received sensitized cells enclosed within millipore chambers impermeable to these cells (Table III). The test homograft was completely destroyed in 6 days or less in 31 of 66 instances and in none of 42 control recipients. The proportion of partial rejections was about the same in both the experimental and control groups. But the extent and severity of the necrosis was much greater in the recipients of sensitized cells than in recipients of non-sensitized cells. In the latter instance (the controls), most of the necrosis was due either to technical difficulties or to early primary rejection. No evidence of rejection was seen in less than one-sixth of the experimental hosts and in two-thirds of the control hosts.

A comparison of A and CBA strains revealed a difference in the immunologic potency of the transferred cells. Sensitized CBA cells enclosed in millipore chambers and placed in CBA hosts rejected completely 21 of 30 A homografts within 6 days. Sensitized A cells in A hosts rejected completely 10 of 36 CBA homografts (Table III). A similar finding was observed in the experiments with transfer of labeled sensitized cells in which 12 of 23 CBA recipients showed complete homograft rejection, and only 4 of 18 A recipients showed complete homograft destruction (Table II).

The region of implantation of the millipore chambers influenced rate of rejec-

tion of the homograft. In mice with subcutaneously implanted chambers containing sensitized cells, there were gross and microscopic signs of rejection by the 3rd day after grafting, and rejection was usually complete by the 6th post-graft day. In mice with intraperitoneal millipore chambers, the gross appearance of all grafts and microscopic appearance of five out of seven biopsies on the 3rd post-graft day showed no signs of rejection. However, by the 6th day after grafting, all grafts elicited both macroscopic and microscopic stigmata of graft rejection.

TABLE III

Transfer of Homograft-Sensitized Cells in Millipore Chambers

Mice		No rejection	Partial rejection or necrosis	Complete rejection
Strain	Number			
A	36	10	16	10
CBA	30	0	9	21
Totals.....	66	10	25	31
		Controls*		
A	18	14	4	0
CBA	24	15	9	0
Totals.....	42	29	13	0

* Transfer of non-sensitized cells in millipore chambers.

Careful examination of the millipore chambers at autopsy of the mice revealed no evidence of perforation of the membranes or separation from the lucite rings. Microscopic examination of the chambers disclosed that no cells or parts of cells were traversing the wall. Within the chambers were clumps and aggregates of viable lymphoid elements and necrotic material. It was not possible by microscopic examination to estimate quantitatively the number of viable cells remaining. There was no apparent histologic difference between the contents of chambers containing isologous sensitized cells and the contents of the control chambers which contained isologous non-sensitized lymphoid cells.

Cells sensitized against A strain tissues and transferred in millipore chambers into the peritoneum of CBA hosts completely rejected 3 out of 6 A skin grafts and had no effect on 6 C3H skin grafts placed on the same CBA recipients.

Intraperitoneal injection of immune serum given as a single 1.67 ml dose on the 2nd day after grafting or as daily 0.67 ml doses for 6 days did not influence homograft survival.

DISCUSSION

This study has demonstrated that sensitized viable lymphoid cells transferred transplantation immunity by rejecting specific homografts within 6 days or less without apparently infiltrating the graft site. These results were in accord with the findings of Mitchison and Dube (6) who, using acriflavine dye as a marker, were unable to find labeled sensitized cells at the site of subcutaneously transferred SAI tumor cells following passive transfer of the sensitized cells. Despite our use of a permanent cellular label such as tritiated thymidine, labeled cells could not be demonstrated at the site of rejection when examined as early as 12 hours after grafting and as late as the 6th post-graft day. Adequate tagging was shown by the 33 per cent average labeling of cells seen on smears of the transferred suspensions, and persistence of the label could be demonstrated by the ease of finding labeled cells in the autoradiographs of spleens and lymph nodes taken 7 days after transfer.

Two explanations may be entertained to explain the results of the passive transfer studies with tritiated sensitized cells. Either the sensitized cells participated in the reaction at the graft site but were rapidly destroyed, or the sensitized cells produced a soluble product which rejected the graft directly or transferred the sensitivity to host cells. The first possibility of rapid destruction of the sensitized cells seemed unlikely since graft sites biopsied at varying time intervals from 12 hours to 6 days after cell transfer failed to disclose labeled cells. In addition, when sensitized labeled lymph node fragments were placed subcutaneously around homografts or directly in contact with the graft, daily biopsy of these nodes revealed a progressive central necrosis of the fragments as would be expected from athrepsia but the outer rim of the fragment remained viable, even on the side in juxtaposition to the rejecting graft.

The series of experiments in which sensitized cells were transferred in millipore chambers indicated that a soluble product was produced by the sensitized cells and this humoral agent could cause rejection of the graft. The question as to whether the humoral agent transferred immunity to the host's cells or acted directly upon the graft could not be answered by these studies.

In this experiment, transfer of homograft immunity could not be accomplished with serum from sensitized animals even when given in doses up to 5 animal equivalents (1.67 ml) in a single intraperitoneal injection or 4 ml administered at the rate of 0.67 ml daily for 6 days. Humoral antibodies detected by hemagglutination (7, 8), leukocyte agglutination (9, 10), and cytotoxicity techniques (11, 12) have frequently been demonstrated in animals rejecting tissue or tumor transplants. However, the isoimmune sera of animals rejecting tissue homografts have generally been ineffective in passive transfer of transplantation immunity (5, 13) unless given locally (14, 15) or given systemically with application of xylene or bromobenzene locally to increase vascular perme-

ability (14). In view of the results of this study, it would appear that the failure to transfer transplantation immunity with serum might be related to the low concentration of "antibodies" present in the sensitized animal at any given time. In our experiments, apparently cells in millipore chambers continuously released a humoral agent in sufficient quantity to reject the homograft.

The question arose as to why sensitized cells within millipore chambers rejected homologous skin grafts in this investigation, in contrast to the reverse situation in which homologous tissues within millipore chambers survived for prolonged periods within sensitized hosts (16, 17). Two explanations for this apparent paradox may be considered. The first is the demonstration of a slow transfer of antibody from the vascular tree to peritoneal cavity as compared to rapid absorption of antibody from the peritoneal cavity to the blood (18). Secondly, Amos has recently shown that complement traverses the millipore membranes with difficulty and he was unable to show titers of complement within the chambers above one-tenth those found in the serum (19). Therefore, inadequate quantities of complement and "antibody" within the millipore chambers might explain why homologous tissues within the chambers have a prolonged survival in sensitized hosts whereas homologous skin grafts were readily rejected when sensitized cells were enclosed within millipore chambers. More recently, Gabourel has reported that growth of L-fibroblasts within millipore chambers was inhibited when the chambers were placed in "hyperimmunized" mice (20).

Finally, these results were distinctly different from those observed in the transfer of tuberculin sensitivity in which the specifically sensitized cells were found in significant numbers in the site tested with specific antigen (3) and were ineffective when enclosed in millipore chambers. However, the test system used for the tuberculin and homograft reactions were accomplished in 2 different species, guinea pigs and mice, respectively. To verify this difference, experiments with transfer of transplantation immunity using tritiated sensitized cells are currently being investigated in inbred guinea pigs.

SUMMARY

Passive transfer of tritiated thymidine labeled lymphoid cells sensitized to homologous tissues into non-sensitized isologous hosts resulted in accelerated rejection of homologous skin grafts in the recipients. Despite 33 per cent label of the suspension, only rare labeled sensitized lymphoid cells could be found at the site of rejection.

Passive transfer of sensitized lymphoid cells in millipore chambers implanted subcutaneously or intraperitoneally in non-sensitized isologous hosts resulted in accelerated homograft rejection in the recipients. Transfer of transplantation immunity could not be accomplished with serum from sensitized hosts.

The rejection of homologous tissues without the physical presence of the sensitized cell at the graft site suggested that a humoral agent produced by the cell was capable of rejecting the homograft.

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