FURTHER STUDIES ON ADOPTIVE TRANSFER OF SENSITIVITY TO SKIN HOMOGRAFTS*

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An important procedure for providing information concerning the site of development of various types of immune response, and the manner in which they are put into effect, involves the passive transfer of immunity to normal individuals. It is now widely believed that the destructive reaction of individuals against vascularized solid tissue homografts is put into effect by immunologically "activated" cells of the lymphocytic series, rather than by humoral antibodies (1, 2). For several years this concept rested principally upon evidence that homograft sensitivity could only be transferred passively (more correctly "adoptively") by means of living cells obtained from the lymph nodes or spleens of specifically immunized animals (3–5). Nevertheless, a well recognized weakness of this thesis was the failure of attempts to transfer sensitivity by means of whole blood, leucocyte concentrates, or peritoneal exudates (3–5). Subsequent evidence that the lymphocyte population of the blood of birds and mammals includes immunologically competent elements (6–9) hinted that this failure may merely have reflected shortcomings of technique, such as lack of sensitivity, rather than the absence of cells capable of transferring immunity. The findings of Gowans *et al.* (9) and our own preliminary observations (10) have shown this to be the case.

This paper presents the results of studies principally designed to compare, on a quantitative basis, the capacity of lymph node cells, whole blood, leucocyte concentrates, thoracic duct lymphocytes and peritoneal exudate cells to transfer skin homograft sensitivity in mice and rats. Additional experiments are reported which were designed to determine the time of appearance and persistence of immunologically "activated" cells, capable of transferring sensitivity, in the regional nodes and blood of recipients of orthotopic skin homografts.

Principle of the Experiments.—There are two not unrelated methods for demonstrating the competence of cellular inocula to transfer homograft immunity adoptively

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between members of an inbred strain (see Fig. 1). In the first method (3, 4) lymphoid cells from donors that have been specifically immunized in respect of the transplantation isoantigens of an alien strain are inoculated into normal, isologous recipients which are subsequently challenged with test-skin homografts from the same foreign strain. Accelerated rejection of these grafts, as a consequence of a "second set" reaction, furnishes evidence of the transfer of sensitivity.

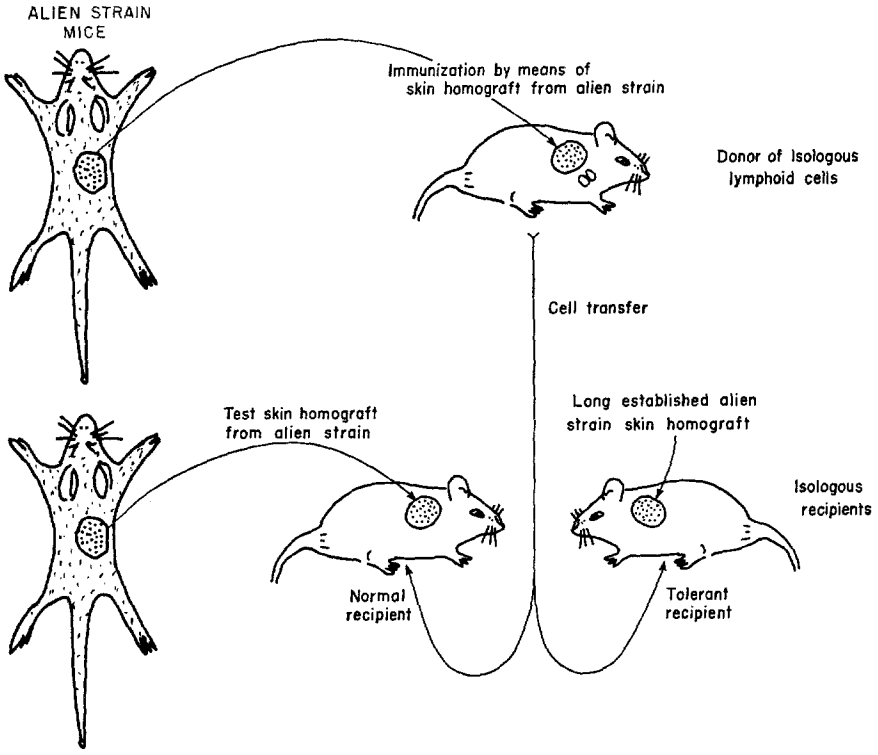

Fig. 1. Illustrating the two methods for demonstrating the competence of cellular inocula to transfer homograft sensitivity adoptively.

The second method (11) requires animals previously rendered specifically tolerant of test skin homografts from the alien donor strain. Abolition of tolerance, as evidenced by destruction of hitherto normal, healthy looking tolerated skin homografts of long standing, can be effected in either of two ways:—(*a*) by transfer of lymphoid cells from a *normal* isologous donor, or (*b*) by transfer of lymphoid cells from an isologous donor previously sensitized in respect of the transplantation antigens of the alien strain. Either way, the immunological shortcomings of the tolerant animal are made good. However, if equal numbers of lymphoid cells from normal and sensitized donors are transferred, those from the latter are more effective as evidenced by the shorter

time required for the abolition of the tolerant state. Furthermore, preliminary studies (10) have shown that this disparity in efficacy becomes greater with reduction in the dosage of cells transferred, until a transfer dosage is reached at which only immunologically "activated" cells have any detectable influence. Cell transfers to tolerant recipients, at a dosage level where normal cells are ineffective, provide the basis of an all-or-nothing test system for indicating the presence of cells capable of transferring homograft sensitivity.

The test system provided by tolerant hosts was employed for most of the experiments to be described, partly because it eliminates the possibility that the sensitivity manifested by these animals could have been caused by their active immunization in response to inadvertently transferred antigen, but principally on account of its superior sensitivity.

Materials and Methods

The subjects of our experiments were animals of both sexes, belonging to domestic isogenic sublines of mouse strains A and CBA, and of the Lewis and B.N. rat strains.

The median survival times, together with their standard errors, of skin homografts exchanged between normal adult animals in the various donor/recipient strain combinations employed were as follows:

Mice: CBA → A	10.2 ± 0.3 days (12)
A → CBA	11.0 ± 0.3 days (12)
Rats: B.N. → Lewis	10.2 ± 0.8 days (13)
Lewis → B.N.	10.0 ± 0.9 days (13)

Some of the tolerant mice used were by-products of an investigation of the tolerance-conferring capacity in newborn mice of different types and dosages of homologous cellular inocula (14). Tolerance was conferred upon the remainder by the intravenous injection at birth with 10 million CBA/A hybrid spleen cells. Tolerant rats were obtained by neonatal intravenous injection with 60 million homologous bone marrow cells.

All neonatally inoculated animals were challenged with donor strain skin grafts when they were 8 to 10 weeks old. Only those which were classified as "highly tolerant", *i.e.* still bore healthy skin homografts with normal fur crops on the 50th postoperative day, were used as hosts for transfer experiments.

Previous work has shown that less than 10 per cent of A strain mice rendered highly tolerant of CBA skin grafts normally lose their tolerant status of their own accord, as evidenced by destruction of their test grafts, during the ensuing 100 days (14, 15). This constitutes the justification for maintaining tolerant animals under close observation for at least 100 days after cell transfer.

Sensitization of isologous donors was accomplished in both mice and rats by means of orthotopic skin homografts transplanted to the right sides of the animals' chests (16), followed in certain designated experiments by the intraperitoneal inoculation of living homologous splenic cells. In a few experiments conducted upon mice an intraperitoneal injection of splenic cells constituted the only immunizing stimulus.

Blood was obtained by cardiac puncture and pooled, using heparin as an anticoagulant. Leucocytes (or, more correctly, leucocyte concentrates, since the preparations inevitably included erythrocytes) were obtained by resuspending the buffy coat of the blood in phosphate-buffered Ringer's solution.

Suspensions of lymph node cells were prepared in bicarbonate-buffered Ringer's solution and

kept at 4°C pending injection. Only the axillary and brachial nodes were used since these are the draining or regional nodes for skin grafts placed upon the lateral thoracic wall (3).

Transfer of cells from sensitized isologous donors to tolerant mice was carried out on the 11th postoperative day if sensitization had been effected by skin homografts; *i.e.*, at a time when destruction of the grafted tissue was almost complete. When splenic cells constituted the only antigenic stimulus, or when they constituted a "booster" stimulus, cell transfers were carried out 8 days after their inoculation since intraperitoneally administered cells are known to evoke sensitivity much more promptly than orthotopic skin grafts (17).

In rats, cells were transferred 10 days after a booster injection of 150 million spleen cells.

Unless otherwise stated, all inocula were transferred to tolerant mice by the intraperitoneal route, and to rats by the intravenous route.

Millipore diffusion chambers were constructed with VC nitrocellulose membranes (Millipore Filter Corp., Bedford, Massachusetts) having a pore size of 100 mμ and a thickness of 130 μ. These were bonded with MF No. 1 cement (Millipore Filter Corp.) onto lucite rings (UVA plexiglass: 14 mm OD × 10 mm ID × 2 mm thickness) each bearing a single access hole. The space within each chamber was 147 mm^3. Before use, the assembled chambers were sterilized with ethylene oxide. After suspensions of cells had been inoculated into these chambers, with the aid of a 24G needle, *via* the access hole, the latter was sealed with duco cement. The loaded chambers were then inserted intraperitoneally in mice *via* a short mid-ventral incision, and 0.5 ml Hanks' solution was introduced into the peritoneum. The abdominal muscle wall was then closed with continuous silk sutures, and the skin with 11 mm Michel wound clips. Not more than 90 minutes elapsed between harvesting the nodes and spleens and the insertion of the chambers intra-abdominally.

Parabiosis in rats and mice was effected by methods described elsewhere (18). The partners were surgically united by their skin and abdominal musculature without a persistent celio-anastomosis. When necessary, separation of the parabionts was performed by severing them at their parabiotic junction, care being taken not to penetrate the abdominal cavity. The wounds were closed with 11 mm Michel wound clips, and healing was complete within 7 days.

Cannulation of the thoracic duct in rats was performed by a modification (19) of the method of Bollman, Cain, and Grindlay (20). After insertion of the cannulae the animals were allowed to recover consciousness after which they were maintained in restraining cages. Lymph was collected overnight into tubes containing Hanks' balanced salt solution (buffered to pH 7.3) and heparin (10 IU/ml).

Cell counts were made with the aid of a hemocytometer after which the concentration of the leucocytes or lymph node cells in the suspensions was adjusted so that the desired cell dosage was administered in a constant volume,—about 1 ml for mice and 2.5 ml for rats.

RESULTS

1. Transfer of Sensitivity in Mice by Means of Lymph Node Cells.—The results of tests carried out with the CBA → A and the A → CBA mouse strain combinations (Tables I and II) fully confirm previous findings that when relatively high dosages of lymph node cells are transferred, cells from *sensitized* donors abolish tolerance more rapidly and consistently than cells from *normal* donors. For both strain combinations there is a cell dosage level at or below which normal node cells are almost, if not completely, incapable of prejudicing the well being of tolerated grafts, whereas cells from *sensitized* donors are still consistently effective. For A strain mice tolerant of CBA grafts this dosage is about 5 million cells, whereas for CBA's tolerant of A tissue, it is about 10 million cells.

With the CBA → A strain combination destruction of a tolerated graft never took place sooner than 8 days after transfer of activated node cells, even when the maximum dosage was employed. Over the dosage range 20 to 240 × 10^6 cells the survival times of the tolerated grafts were remarkably uniform. However, with cell dosages below 20 × 10^6 tolerance was abolished less rapidly in a significant proportion of the subjects.

TABLE I

Abolition of Tolerance of CBA Skin in A Strain Mice by the Intraperitoneal Transfer of Isologous Node Cells

Isologous donors		No. of cells transferred (× 10^6)	Tolerant A strain mice		
Status	Immunizing stimulus		No. tested	Survival times of grafts after cell transfer	MST*
				days	*days*
Normal	—	240	3	12 (2)‡, 14	12
		120	3	20, >100 (2)	
		30	6	>100 (6)	
		15	5	18, 19, 56, >100 (2)	
		5	6	>100 (6)	
Sensitized§	CBA skin	240	5	9 (4), 10	9
		120	5	8 (3), 10 (2)	8
		60	8	9 (4), 10 (4)	9
		30	6	11 (3), 14, 17, 25	12
		20	5	9 (5)	9
		15	8	10 (2), 11 (2), 17, 18, 22 (2)	12
		10	8	10, 11 (3), 24, 25, 55, >100	12
		5	12	15 (2), 16, 17, 19 (2), 20, 24, 25, 39 (2), 45	21

* Median survival time.

‡ Numbers in parentheses indicate No. of animals.

§ Suspensions of regional node cells were prepared from sensitized donors 11 days after they had been grafted with CBA skin.

2. Transfer of Immunity in Mice by Means of Leucocytes.—The tests conducted with various dosages of leucocytes transferred from *normal* animals (Tables II and III) indicated that, with both mouse strain combinations, the tolerance-abolishing capacity of these cells is comparable to that of node cells. With the CBA → A combination (Table III), *leucocytes*, transferred from donors 8 days after they had received a booster injection of homologous spleen cells, were just as effective in abolishing tolerance as cells from the regional nodes of donors grafted with homologous skin 11 days beforehand. It can be seen that leucocytes from donors immunized by skin followed by spleen were not perceptibly more effective than leucocytes from donors immunized by

means of a single intraperitoneal injection of homologous spleen. This suggests that the circulating leucocytes of animals which have just rejected primary skin homografts express the near maximal level of sensitivity evokable by any regimen of immunization.

The finding that as few as 0.63×10^6 leucocytes from sensitized donors abolished tolerance in most of the A strain subjects provides some indication of the sensitivity of the test system.

TABLE II

Abolition of Tolerance of A Strain Skin in CBA Mice by the Intraperitoneal Transfer of Isologous Node Cells or Leucocytes

Type of cell transferred	Status of isologous donors*	No. of cells transferred ($\times 10^6$)	Tolerant CBA mice		
			No. tested	Survival times of tolerated A strain grafts after cell transfer	MST
				days	*days*
Node	Normal	20	6	60, 90, >100 (4)	
	Normal	10	6	40, >100 (5)	
	Normal	5	3	>100 (3)	
	Sensitized*	10	6	12, 13 (2), 18, 19, 27	13
	Sensitized*	5	8	22, 32 (2), 34, 46, 84, >100 (2)	34
Leucocytes (buffy coat)	Normal	20	6	45, 48, >100 (4)	
	Normal	10	5	>100 (5)	
	Normal	5	6	>100 (6)	
	Sensitized*	10	6	14, 17, 18, 20, 22, 27	19
	Sensitized*	5	5	14, 15, 19, 21, 29	19
Leucocytes (in whole blood)	Sensitized*	5	8	11, 14 (2), 15 (2), 17, 24, >100	15

* Isologous donors were sensitized by means of A strain skin homografts. Cells were transferred from these animals on the 11th postoperative day.

Comparison of the tolerance-abolishing capacity of 5 million leucocytes present in a resuspension of the buffy coat in Ringer-phosphate, with that of whole blood containing a similar number of leucocytes, indicates that these cells are equally effective in both types of inocula. Evidently the presence of plasma, containing humoral isoantibodies, makes no demonstrable contribution to the effectiveness of leucocytes in transferring sensitivity—a finding entirely consistent with previous failures to abolish tolerance by transfer of putatively immune sera, even in massive amounts (21, 22).

As the dosage of "immune" leucocytes transferred to the A strain hosts was

reduced not only did the minimum survival times of the tolerated grafts increase, but the range of survival times broadened, some of the grafts surviving throughout the observation period.

Throughout the experiments with mice all cells were transferred *via* the

TABLE III

Abolition of Tolerance of CBA Skin in A Strain Mice by the Intraperitoneal Transfer of Isologous Leucocyte Concentrates

Isologous donors		No. of cells transferred ($\times 10^6$)	Tolerant A strain mice		
Status	Immunizing stimulus		No. tested	Survival times of grafts after cell transfer	MST
				days	*days*
Normal	———	30	3	15, >100 (2)	
		15	7	14, 15, 16, 27, 45, 73, >100	23
		10	6	25, >100 (5)	
		5	10	>100 (10)	
Sensitized*	CBA skin followed by intraperitoneal injection of 50×10^6 CBA spleen cells	30	6	8, 11, 12, 13, 14, 15	13
		15	7	11 (5), 12, 20	11
		10	6	14, 15, 16, 18, 20 (2)	17
		5	16	11 (3), 13 (2), 14, 16, 19, 21, 22, 26, 27, 32, 40, >100 (2)	20
		2.5	8	18, 25, 28, 29, 43, 44, 75, >100	36
		1.25	11	17 (2), 18, 19, 22, 72, 96, >100 (4)	40
		0.63	7	26, 28, 37 (2), 54, >100 (2)	36
Sensitized*	50×10^6 CBA spleen cells injected intraperitoneally	10	2	13, 14	
		5	3	14 (2), 15	
Sensitized	CBA skin	5	7	11, 13 (4), 15, 16	13
		5‡	6	13 (4), 14, > 100	13
		5§	6	18, 24, 34, 97, > 100 (2)	~30

* Leucocytes were transferred from these animals 8 days after inoculation of spleen cells.

‡ Aliquots of whole blood containing 5 million leucocytes were transferred intraperitoneally.

§ Aliquots of whole blood containing 5 million leucocytes were transferred *via* the tail vein.

intraperitoneal route principally as a matter of technical convenience. To ensure that this entailed no unnecessary loss of sensitivity on the part of the test system, a group of 6 tolerant A strain animals were injected intravenously, *via* the tail vein, with 1 ml of heparinized blood containing 5 million leucocytes, from an isologous donor immunized by means of a skin homograft. So far as they go the results, presented in the final line of Table III, suggest that this route may

be slightly less effective than the intraperitoneal one. It is of interest that, as with intraperitoneal cell transfers, tolerance was not abolished in some of these hosts during the 100 day observation period.

TABLE IV

Appearance and Persistence of Cells Capable of Transferring Transplantation Immunity in Regional Nodes and Blood of A Strain Mice Grafted with CBA Skin

Day of transfer of cells from immunized isologous donors	Transfer experiments with node cells			Transfer experiments with leucocytes		
	No. of tolerant hosts injected*	Survival times of tolerated grafts after cell transfer	Grafts rejected	No. of tolerant hosts injected*	Survival times of tolerated grafts after cell transfer	Grafts rejected
		days	*per cent*		*days*	*per cent*
4	6	>100 (6)	0	6	>100 (6)	0
5	6	>100 (6)	0	7	>100 (7)	0
6	8	17, 23, 25, 26, 30, >100 (3)	62	8	17, 28 (2), 29, 36, >100 (3)	62
8	9	14 (2), 34, 42 (2), 83, >100 (3)	67	9	24, 48, 80, 81, >100 (5)	44
11	12	15 (2), 16, 17, 19 (2), 20, 24, 25, 39 (2), 45	100	15	11, 13 (8), 14, 15, 16, >100 (3)	80
20	6	39, 64, >100 (4)	33	6	14, 15, 21, 22, >100 (2)	67
40				6	12, 16, 17, 19, 22, 25	100
80	8	9, 10, 11, 80, 90, >100 (3)	62	9	12, 14, 15, 20, 27, 53, 70, >100 (2)	78
120	6	11, 13, 14, 17, 26, >100	83	8	28, 40, 67, >100 (5)	38
165	6	40, 51 (2), 62, >100 (2)	67	5	30, 34 (2), 67, >100	80
200	6	50, 53, 58, 60, >100 (2)	67	9	28, 42, 90, >100 (6)	33
250	5	40, >100 (4)	20			
370	5	60 (2), 80, >100 (2)	60	5	31, 77, 85 (2), >100	80

* Each tolerant A strain mouse received a standard intraperitoneal inoculation of 5 million node cells or leucocytes from isologous donors previously immunized with CBA skin.

3. Time of Appearance and Persistence of Cells Capable of Transferring Sensitivity in Regional Nodes and Blood of Mice Grafted with Homologous Skin.—The finding that transfer of 5×10^6 activated regional node cells or blood leucocytes almost consistently caused rejection of CBA grafts on tolerant A strain hosts, whereas the same dosages of cells from *unsensitized* donors were ineffective, provided the basis of a test for the presence of immunologically activated cells in nodes and blood at various times after exposure to skin homografts.

Panels of normal A strain mice were grafted with CBA skin. At various inter-

vals thereafter blood was collected by cardiac puncture, heparinized, and then pooled. The regional nodes were also removed from these animals, pooled, and cell suspensions prepared therefrom. Aliquots of pooled blood containing 5 million leucocytes, or of regional node cell suspensions containing the same number of cells were then injected intraperitoneally into tolerant A strain mice. In the light of the previous data, rejection of a reasonable proportion of the hitherto tolerated grafts within 100 days of cell transfer may be taken as evidence of the presence of immunologically activated cells in the inoculum. Furthermore, the interval between cell transfer and breakdown of the tolerated grafts provides some indication of the residual level of sensitivity in the donors at the time of cell transfer.

The results of this investigation (Table IV) indicate that not until the 6th day after A strain mice have received skin homografts are detectable numbers

TABLE V

Per Cent Epithelial Survival in CBA Skin Homografts of 6 Days' Standing Transplanted to A Strain Hosts

(*a*) Not previously grafted	100 (6)
(*b*) Grafted with CBA skin 370 days previously	25, 50 (3)

of "activated" cells present in their nodes and blood. At this time both leucocytes and regional node cells seem to be equally effective in transferring sensitivity.

Although there is considerable variation between the results of the individual time lapse tests, taken as a whole the findings show that "activated" cells, capable of transferring homograft sensitivity, are still demonstrable in both the regional nodes and the blood of animals sensitized *more than a year* after they had received skin homografts. Furthermore, the level of the sensitivity transferable by 5 million node cells or leucocytes seems to die away very slowly following this active immunization in response to skin homografts. Confirmation of the persistence of sensitivity in A strain mice, immunized with CBA skin 370 days beforehand, was obtained by challenging a panel of four of such animals with "second set" CBA skin grafts and removing them on the 6th postoperative day for histological evaluation of their epithelial survival scores (Table V).

4. Studies on Sensitivity Induced by Injection of A strain Mice with a Low Dosage of CBA Spleen Cells.—Although dissociated suspensions of splenic cells injected intraperitoneally elicit sensitivity more rapidly than orthotopic skin grafts, the sensitivity evoked by the latter is of much greater duration (17, 22, 23). Experiments have been carried out to determine whether these differences, resulting from differences in mode of immunization, are reflected in the capacity of leucocytes to transfer homograft sensitivity.

A group of A strain mice were immunized by the intraperitoneal inoculation of a standard dosage of 5 million CBA spleen cells; this number of cells is unlikely to be inferior to that present in skin homografts of the size employed to sensitize mice,—discs of skin about 1.0 to 1.2 cm in diameter. Some of these mice were bled 5 days, 11 days, or 80 days after immunization and aliquots of their pooled blood, containing 5 million leucocytes, were injected intraperitoneally into panels of tolerant A strain hosts. Table VI presents the results of these tests together with those previously obtained by transfer of leucocytes from mice immunized with orthotopic skin homografts.

Upon comparison, it can be seen that whereas immunologically activated cells are present earlier in the blood of mice immunized with suspensions of

TABLE VI

Comparison of Sensitivity-Transferring Capacity of Leucocytes from A Strain Mice Sensitized with Grafts of CBA Skin with That of Leucocytes from Mice Sensitized by Means of 5 Million CBA Spleen Cells Inoculated Intraperitoneally

Isologous donors		Tolerant A strain mice	
Antigenic stimulus administered	Day of leucocyte transfer after immunization	No. tested	Survival times of tolerated CBA grafts after leucocyte transfer
			days
Skin	5	7	>100 (7)
Spleen cells		6	19, 80, 84, >100 (3)
Skin	11	15	11, 13 (8), 14, 15, 16, >100 (3)
Spleen cells		6	18, 38 (2), 45 (2), >100
Skin	80	9	12, 14, 15, 20, 27, 53, 70, >100 (2)
Spleen cells		6	70 (3), 77, 84, >100

splenic cells, cells with a greater capacity to transfer sensitivity persist longer in mice immunized with skin homografts. These results may be taken as corroborative evidence that immunization with an orthotopic skin graft leads to a stronger and more persistent level of homograft immunity than immunization with splenic cells inoculated intraperitoneally.

5. Transfer of Sensitivity with Peritoneal Exudate Cells.—

To test the ability of peritoneal exudate cells to transfer sensitivity, normal A strain hosts were grafted with CBA skin. On the 11th day, each animal was injected intraperitoneally with 2 ml of mineral oil. Then 2 days later, each was injected intraperitoneally with 3 ml of citrate-saline. Some of these animals were bled to test the sensitivity-transferring capacity of their leucocytes. Finally, the peritoneal fluid was collected, pooled, and the oil phase discarded (4). The cells suspended in the aqueous phase were then counted. Groups of tolerant A strain hosts were injected intravenously with (*a*) 5 million or (*b*) 10 million peritoneal exudate cells, or (*c*) blood containing 5 million leucocytes obtained from some of these animals.

The results (Table VII) show that, contrary to previous findings, sensitivity is transferable by means of peritoneal exudate cells, though apparently less effectively than by leucocytes or regional node cells. This disparity probably reflects the fact that a much higher proportion of cells of the lymphocytic series are present in blood leucocytes than in peritoneal exudate cells. According to Mitchison (4) 50 to 60 per cent of the latter are mononuclear cells. Estimates of the proportion of lymphocytes present in mouse blood leucocytes range from 60 to 80 per cent (24).

It may be noted that leucocytes from sensitized animals that have received mineral oil intraperitoneally appear to be less effective in abolishing tolerance than similar cells from animals which had not been inoculated with this sub-

TABLE VII

Transfer of Sensitivity to Tolerant A Strain Mice by Means of Peritoneal Exudate Cells

Type of cell transferred	No. of cells transferred	No. of tolerant mice tested	Survival times of tolerated grafts after cell transfer
	millions		*days*
Peritoneal exudate	5	11	44, 58, 68, >100 (8)
Peritoneal exudate	10	6	30, 33, 45, >100 (3)
Leucocytes*	5	6	33 (2), 59, 66, >100 (2)

* These leucocytes (in whole blood) were obtained from animals sensitized with skin homografts and injected intraperitoneally with mineral oil on the 11th postoperative day to facilitate collection of the peritoneal exudate cells 2 days later (see text).

stance (see Table III). Although the possibility cannot be excluded that this may be a sampling error, this difference may be indicative of a disturbance in the normal differential leucocyte count.

6. Transfer of Sensitivity with Contralateral Lymph Node Cells.—It is well established that cells from the contralateral axillary and brachial nodes are very much less effective in transferring actively acquired homograft sensitivity of fairly recent origin than cells derived from regional nodes (3). To extend this work, tests were conducted in which contralateral node cells were transferred to isologous tolerant hosts from A strain donors at various intervals after the latter had been grafted with CBA skin. The results (Table VIII) indicate that, whereas contralateral node cells removed from donors at the median survival time of their immunizing grafts are only just perceptibly capable of transferring sensitivity, cells obtained from these nodes at longer intervals after the donors had received skin homografts are more effective.

To determine whether the presence of the regional nodes in actively immunized mice plays an important role in the maintenance of immunologically activated cells in the blood stream, eight A strain mice were grafted with CBA skin on the right sides of their chests. On the 28th postoperative day the regional axillary and brachial nodes were carefully excised. 54 days

later these animals were bled and aliquots of the pooled blood containing 5 million leucocytes were injected intraperitoneally into 5 tolerant A strain mice bearing healthy CBA skin homografts.

The survival times of these grafts after cell transfer were 20, 23, 44, 46, 53 days, respectively, indicating that the excision of the regional nodes from the sensitized donors did not seriously impair their ability to maintain an effective level of "cellular" immunity in their blood.

These findings sustain the conclusion that although the regional nodes are the first lymphoid organs to be activated by the transplantation of orthotopic skin homografts, activated status is ultimately acquired by other nodes and by the spleen. Although this could be due to the progressive wider distribution of

TABLE VIII

Transfer of Homograft Sensitivity from Secondary A Strain Hosts, Grafted with CBA Skin, to A strain Mice Tolerant of CBA Skin by Means of Cells from the Contralateral Lymph Nodes

Interval between grafting isologous donors with CBA skin and cell transfer	Tolerant A strain mice*	
	No. tested	Survival times of tolerated grafts
days		*days*
11	8	27, 49‡ 84 (2), 94, >100 (3)
20	6	39, 64, 98 (2), >100 (2)
40	6	39 (2), 50, >100 (3)
60	6	21, 24, 35, 42, 56, >100
80	5	27, 46, 78, 95, >100

* Each tolerant mouse received a suspension of 5 million node cells intraperitoneally.

‡ Animal died 49 days after cell transfer.

an antigenic stimulus emanating from the graft, a more probable explanation is that it reflects the spread of sensitized cells from one lymphoid center to another (*cf.* reference 2). This interpretation is sustained by the evidence that activated cells are present in both leucocytes and peritoneal exudates.

7. Influence of Splenectomy on Sensitization of Secondary Donors.—It is well established that splenectomy does not weaken the vigor with which animals react against skin homografts (25, 26). However, it has been shown that prior splenectomy enhances the capacity of lymphoid cells from rats sensitized with spinal cord plus Freund's adjuvant to transfer experimental allergic encephalomyelitis to normal rats (27, 28). In the light of these considerations the influence of prior splenectomy on the capacity of isologous donor node cells or leucocytes to transfer homograft sensitivity has been studied.

A strain mice were splenectomized and 100 days later sensitized with CBA skin grafts. On the 11th postoperative day they were bled and their regional nodes excised. Aliquots of

pooled blood, containing 5 million leucocytes, or of a pooled suspension of regional nodes containing this number of cells were injected intraperitoneally into two groups of tolerant A strain hosts. The survival times of the tolerated grafts on these animals are presented in Table IX.

The finding that leucocytes from splenectomized donors abolished tolerance *less* rapidly than leucocytes from donors whose spleens were intact suggests that the spleen does in fact make a substantial contribution to the sensitized cell population of the blood of mice grafted with homologous skin (*cf.* reference 3). It is difficult to explain why regional node cells from splenectomized animals were also less effective than those from donors whose spleens were intact, and to reconcile these findings with those on the transferability of allergic

TABLE IX

Effect of Prior Splenectomy on Capacity of A Strain Mice to Produce Activated Cells in Their Regional Nodes and Blood Following Subsequent Immunization by Means of Orthotopic CBA Skin Homografts

Type of cell transferred*	Splenectomized	No. of tolerant mice tested	Survival times of tolerated grafts *after* cell transfer
			days
Leucocytes	No	15‡	11, 13 (8), 14, 15, 16, >100 (3)
Leucocytes	Yes	7	18, 29, 64, 70, 80, 95, >100
Regional nodes	No	12‡	15 (2), 16, 17, 19 (2), 20, 24, 25, 39 (2), 45
Regional nodes	Yes	6	20 (2), 42, 52 (2), 85

* A standard dosage of 5 million node cells or leucocytes was transferred.

‡ These results, which constitute controls, have been transcribed from Tables IV and I, respectively.

encephalomyelitis from splenectomized and subsequently sensitized rats (27, 28).

8. Attempt to Abolish Tolerance by Means of Activated Node Cells Transferred in Cell-Impermeable Millipore Chambers.—According to recent findings of Najarian and Feldman (29, 30) transfer of relatively high dosages of lymphoid cells, in cell-impermeable Millipore diffusion chambers, from A strain mice actively immunized against CBA tissue, to normal A strain mice, confers heightened sensitivity upon the latter in respect of subsequent grafts of CBA skin. In view of the potential significance of this work, it was decided to employ this method to try and procure rejection of CBA grafts borne by tolerant A strain hosts.

Following Najarian and Feldman's (29) procedure closely, a group of A strain mice were grafted bilaterally with CBA skin and 2 days later injected intraperitoneally with 10 million CBA splenic cells. Nine days after these animals had received skin homografts, their spleens,

axillary, brachial, and cervical nodes were excised, pooled, and dissociated in phosphate-buffered Ringer's solution containing 20 per cent PVP w/v (K-30; Antara Chemicals, Philadelphia) to produce a cell suspension. Aliquots of 0.12 ml of this suspension, containing about 200 million lymphoid cells, were introduced into a series of sterile Millipore chambers which were then carefully sealed. Two chambers were then inserted into the body cavities of each of five tolerant A strain mice.

The presence of this high dosage of sequestered "immune" lymphoid cells produced no discernible adverse changes in the tolerated CBA grafts during a 54 day observation period. Finally, therefore, each tolerant mouse was injected intraperitoneally with 20 million regional node cells from an A strain mouse which had been grafted with CBA skin 11 days beforehand. The complete destruction of all five tolerated grafts within 8 to 9 days ruled out any possibility of questioning their susceptibility to immunity of adoptive origin.

TABLE X

Survival Times of CBA Test Homografts on Normal A Strain Mice Following Transfer of Lymphoid Cells in Diffusion Chambers from Immunized or Normal Isologous Donors

Immunological status of transferred cells	No. of animals tested	Survival times of test skin homografts
		days
Sensitized	11	10, 11 (3), 12 (4), 13, 14, 15
Normal	3	10, 13, 14

Because of this failure to transfer sensitivity to tolerant mice by means of activated lymphoid cells in diffusion chambers, an attempt was made to use almost exactly the same experimental design as that of Najarian and Feldman, to see whether sensitivity to CBA skin could be transferred to normal A strain mice by means of activated lymphoid cells in Millipore chambers.

Anti-CBA lymphoid cell suspensions were prepared from A strain mice as described above, and aliquots of 200 million cells were placed in chambers as before. Two loaded chambers were then inserted intraperitoneally into a group of normal adult A strain mice. To provide controls, some A strain mice received chambers containing similar numbers of lymphoid cells from *unsensitized* A strain animals. Two days after implantation of the chambers all the mice were challenged with CBA skin. The dressings were removed and primary inspection of the grafts was carried out on the 6th postoperative day. Grafts were subsequently inspected at daily intervals, to establish their survival times.

Both series of grafts healed in perfectly. The well being of the grafts borne by the putatively sensitized hosts on the 6th postoperative day, and their survival times (Table X) indicated that no effective sensitivity was transferred under the conditions of these experiments.

9. Transfer of Homograft Sensitivity by Parabiosis.—The first successful transfers of homograft sensitivity, in respect of tumor tissue homografts, were

procured by parabiosis of mice (*cf.* reference 4, 31, 32). Unfortunately, this technique was incapable of revealing the nature of the resistance factors involved. Since parabiosis has also been employed to induce tolerance of tissue homografts, and even to "transfer" an established tolerant state from one animal to another of similar genetic constitution (*cf.* reference 33), transfer of skin homograft sensitivity to normal or to tolerant hosts by parabiosis was investigated.

TABLE XI

Transfer of Homograft Sensitivity by Parabiosis

Strain of test subjects	Tolerant of:—	Immunological status of isologous partner	Duration of parabiosis	Interval between separation of parabionts and test grafting	No. of test subjects	Survival times of test grafts
			days	*days*		*days*
CBA	—	Immune	11	7	5	8, 9 (3), 11
CBA	—	Immune	17	8	8	7 (6), 8 (2)
CBA	—	Immune	25	8	5	6 (3), 7 (2)
A	—	Immune	9	8	7	6 (5), 8 (2)
A	—	Immune	11	42	5	7, 8 (2), 9 (2)
A	CBA	Normal	—*	—*	5	11, 15, 17 (3)
A	CBA	Immune	—	—	2	12, 13
CBA	A	Normal	—	—	10	16, 20 (3), 22 (2), 25, 31, 36, >50
CBA	A	Immune	—	—	10	13 (9), 17
B.N.	Lewis	Normal	—	—	6	20, 21, 25, 28, 29, >50
Lewis	B.N.	Normal	—	—	5	11, 12, 13, 15 (2)

* Tolerant animals were maintained in parabiotic union until rejection of hitherto tolerated grafts took place.

Transfer of sensitivity was studied in two different ways. The first, employing the classic procedure, simply entailed uniting a normal test subject to an isogenic, presensitized partner which had received a skin homograft 15 to 16 days previously. After 11 to 25 days the parabionts were separated and about a week later, after the lesions had healed, the test animal was challenged on its unoperated side with a skin homograft from the strain in respect of which its partner had been sensitized. Accelerated rejection of the skin graft furnished evidence of the transfer of sensitivity to the normal partner. The second method of studying transfer of sensitivity by parabiosis required the use of tolerant animals. With these, evidence of the transfer of sensitivity depended upon showing that tolerated grafts were rejected *more* rapidly following union to sensitized isologous partners than to normal ones.

Table XI summarizes the various experiments carried out. When normal CBA mice were united to presensitized partners they did not acquire maximal sensi-

tivity to A strain skin unless the duration of parabiosis exceeded 17 days. Eleven days was insufficient. Transfer of sensitivity in respect of CBA tissue in A strain hosts occurred more rapidly since with this combination 9 days union sufficed. Furthermore, evidence was obtained with A strain mice that sensitivity acquired through parabiosis dies away fairly rapidly (compare survival times of test grafts on animals grafted 8 and 42 days after severance of their parabiotic unions).

As anticipated, parabiosis of tolerant mice or rats with *normal* isologous partners resulted in destruction of their tolerated grafts. This was probably the combined result of two concomitant processes.—(*a*) the re-equipment of the tolerant animal with a normal, *functional* cellular machinery of immunological response, furnished by immunologically competent cells "abstracted" from the partner's blood stream, and (*b*) primary sensitization of the normal parabiont as a consequence of its presumed chronic perfusion with foreign cells derived from the chimeric leucocyte population of the tolerant animal, followed by transfer of some of the resultant immunologically activated cells *via* the blood.

Tolerance was abrogated more rapidly when tolerant hosts were united to presensitized partners, presumably as a consequence of the transfer, *via* established vascular anastomoses, of activated lymphoid elements.

10. Abolition of Tolerance of Skin Homografts in Rats by Transfer of Cells from Normal or Sensitized Donors.—Although the experiments carried out on mice show that skin homograft sensitivity is transferable by lymph node cells, leucocytes, or peritoneal exudate cells, they do not identify the cell type responsible.

To make good this shortcoming, it was necessary to turn to the rat. Small scale trials with both the Lewis → B.N. and the B.N. → Lewis combinations confirmed that both node cells and leucocytes transferred from *sensitized* donors abolished tolerance more rapidly than similar cells from normal donors, and provided a useful indication of the order of magnitude of the cell dosages required for each combination. Lewis rats tolerant of B.N. skin seemed to require the transfer of fewer lymphoid cells to abolish tolerance than B.N. rats tolerant of Lewis skin and were consequently selected for the definitive tests.

The particular importance of the rat for this investigation is the facility with which its thoracic duct can be cannulated to furnish lymph containing almost exclusively lymphocytes (about 95 per cent of the small variety (34)). These cells are normally destined to enter the blood stream, maintaining its lymphocyte level through their constant recirculation from blood to lymph *via* the lymph nodes (35, 36).

When the tolerance-abolishing capacity of suspensions of thoracic duct lymphocytes from normal and immunized donors was compared (Table XII), using Lewis hosts tolerant of B.N. skin grafts, it was found that even at dosage

levels as low as 10 million cells normal lymphocytes were still quite effective in abolishing tolerance. However, the finding that at dosages of 10, 20, and 50 million cells, those from *non-sensitized* donors were somewhat less effective,

TABLE XII

Abolition of Tolerance of Skin Homografts in Rats by Transfer of Leucocytes, Node Cells, or Thoracic Duct Cells

Strain combination	Cells transferred		Results (a) With cells from normal isologous donors		Results (b) With cells from sensitized isologous donors	
	Type	No. ($\times 10^6$)	No. of tolerant rats tested	Survival times of tolerated grafts	No. of tolerant rats tested	Survival times of tolerated grafts
				days		*days*
Lewis → B.N.	Leucocytes	500	3	15, 20 (2)		
		250	7	37, >100 (6)	2	12, 40
		150	7	26, >100 (6)		
		75	4	>100 (4)	5	19, 46, 70, >100 (2)
	Nodes	300			2	15 (2)
		150			3	17 (3)
		75	4	23, 24, 30, 32	5	15, 17 (3), 25
B.N. → Lewis	Leucocytes	50 to 70	3	14, 15, 24	4	11 (2), 12, 13
		7.5	3	72, >100 (2)	4	55, >100 (3)
	Nodes	20			3	21 (3)
		10			3	21 (3)
		5			3	21, 28, 29
	Thoracic duct	70 to 90	4	14, 18, 23, 25		
		50	5	13 (2), 19, 34, 42	10	13 (3), 16 (2), 21 (2), 28 (2), >100
		20	2	25, 28	5	19 (3), 20 (2)
		10	4	25, 28, 29, >100	9	19, 20 (2), 21, 25 (2), 40, 54, >100
		5	3	>100 (3)	6	37 (3) >100 (3)
		2.5			3	70, >100 (2)

In these experiments all cells were transferred by the intravenous route.

and that at lower dosages only *sensitized* cells were effective, indicates that the lymphocyte is the predominant, if not the exclusive, cell present in blood which is responsible for its ability to transfer homograft sensitivity (*cf.* reference 9).

It need hardly be emphasized that the ability of relatively low dosages of isologous thoracic duct lymphocytes from *normal* donors to abolish tolerance in

isologous hosts is just as valid evidence that these include immunologically competent cells as the ability of thoracic duct cells to cause runt disease on inoculation into infant homologous hosts (8, 13) or adult F_1 hosts (37).

DISCUSSION

The various findings reported in this paper show that immunologically tolerant mice and rats, bearing "indicator" skin homografts of long standing, are superior in several important respects to normal animals for studies on the adoptive transfer of transplantation immunity. Most important is their sensitivity as test subjects, since destruction of hitherto tolerated homografts may be complete within 2 to 3 weeks, or less, following transfer of a few million node cells or leucocytes from actively immunized donors. On the other hand, transfer of sensitivity to *normal* hosts, recognizable in terms of the accelerated destruction of "second set" test homografts, requires the transfer of what may be considered as excessive numbers of activated cells, as Winn (38) has emphasized.

However, it must not be overlooked that when low dosages of "activated" lymphoid cells are transferred, either intraperitoneally or intravenously, from a common pool to a group of tolerant animals, the subsequent survival times of their grafts may vary within very wide limits. Quite frequently the grafts on some animals remain completely unaffected, or may survive a chronic display of outward signs of a more or less ineffective level of sensitivity, such as partial contracture and alopecia, throughout the standard 100 day observation period. The following factors may contribute to this variability in results:—(*a*) With intraperitoneal injections a variable amount of the cellular inoculum may sometimes be accidentally deposited in the lumen of the gut, or in some site less favorable for the continued viability, dissemination, and expression of the immunological potentialities of the cells (39) than that afforded by the peritoneal cavity. That this possibility is rather remote is suggested by the variable results also obtained when low dosages of activated cells were transferred by the intravenous route. (*b*) Highly tolerant animals are chimeric with respect to their myeloid, and lymphoid tissues and their leucocytes, so that the skin homografts they bear comprise only *part* of the total "target" cell population confronting the transferred cells (11, 40). Since our mice had been rendered tolerant by neonatal inoculation with various dosages of *different* cell types, they may have differed considerably with respect to both the distribution and numbers of alien cells in their various tissues. This may be important in so far as there is probably some relationship between the number of cells transferred and the quantity of target cells they can destroy, at least within a constant time. That the level of cell chimerism can influence the facility with which transferred cells can bring about the destruction of hitherto tolerated skin homografts is suggested by recent findings of Michie and Woodruff (41). The consistently prompt

rejections procured by transfer of large numbers of cells also supports this view, as do Winn's (42) findings with an "adoptive transfer" technique in which known dosages of "target" tumor cells and specifically "activated" lymphoid cells are mixed and then inoculated subcutaneously into appropriate hosts. (*c*) Where low dosages of immune lymphoid cells are transferred to animals with a high degree of cell chimerism, the abundance of antigen confronting them may even be sufficient to procure immunological unresponsiveness on the part of the putative attacking cell population.

The possibility must also remain on probation that the existence of some degree of cell chimerism may actually underlie the great facility with which tolerant animals express immunity of adoptive origin. It ensures exposure of the transferred cells to an additional antigenic stimulus almost immediately after their introduction into the tolerant host (*cf.* reference 14). Evaluation of this possibility requires studies on transfer of sensitivity to *non*-chimeric hosts bearing vascularized "target" tissue homografts against which they are incapable of reacting. These include adult mice which have been rendered immunologically unresponsive to skin homografts through neonatal thymectomy (43), and Syrian hamsters bearing homografts in their immunologically privileged cheek pouches, or bearing healthy cheek pouch skin homografts of long standing on their chests (44).

There is nothing inconsistent about the fact that a test skin homograft upon an adoptively immunized *normal* host may be totally destroyed within 6 days, as a consequence of a "second set" type reaction, whereas even when large numbers of activated cells are transferred to tolerant hosts, their grafts are rarely destroyed before the 9th day. There is a wealth of evidence that healed-in, well established grafts are less vulnerable to sensitivity than freshly transplanted ones (*cf.* reference 45).

The finding that cells capable of transferring homograft sensitivity are first demonstrable in the blood and regional nodes of A strain mice 6 days after they have received homografts of CBA skin has two important implications. Firstly, since orthotopic skin homografts usually elicit histologically detectable changes in the regional nodes, indicative of the inception of an immunological response, within 4 days (46, 47), at least 2 additional days appear to be necessary for the production of detectable numbers of activated cells. Secondly, the appearance of activated cells in the nodes and blood at approximately the same time suggests that these cells must be liberated into the blood very soon after their formation in the nodes. These facts, in conjunction with the finding that "activated" cells are demonstrable in the blood for hundreds of days after mice have rejected skin homografts, may be considered to represent additional circumstantial evidence in favor of the thesis that the destruction of vascularized, solid tissue homografts, is mediated by blood-borne cells. Moreover, the almost indefinite persistence of "activated" cells in the blood of mice immunized by

means of skin homografts may be considered to provide a firm basis for the belief that the "second set" reaction is predominantly the outcome of a *pre-existing* state of sensitivity (2). Although the occurrence of a secondary or anamnestic response to second set homografts, heightening the level of the pre-existing sensitivity, almost certainly occurs, it probably fails to intervene soon enough to affect such grafts (23).

A sound physiological basis for the long persistence of activated cells in the blood stream of sensitized animals is provided by Gowans' (34, 35) evidence that lymphocytes are long lived cells, probably with a life span of the order of 100 days, and that these cells undergo a continuous recirculation from blood to lymph, probably *via* the lymph nodes and efferent lymphatics.

Although it is well established that the majority of the infiltrating small lymphocyte population in homografts borne by normal hosts, or in skin homografts borne by tolerant hosts rehabilitated by transfer of cells from normal isologous donors, are of *new* formation (36, 48), unequivocal evidence that these cells are the causal agents of graft destruction has yet to be produced. However, recent studies indicate that suspensions of activated lymphoid cells, including thoracic duct cells, are capable of destroying homologous target cells *in vitro* (49, 50). So far attempts to demonstrate, by employment of isotopically labeled cells, that the infiltrating mononuclear cells in skin homografts on adoptively immunized animals are either transferred cells, or their descendants, have been unsuccessful (29, 51). Studies on the adoptive transfer of delayed hypersensitivity to various allergens in guinea pigs, by means of isotopically labeled lymphoid cells, have, with one exception (52), also failed to produce evidence of specific localization of marked cells at antigen challenge sites (*cf.* references 33 and 53).

The present studies shed very little light on the fate of the cells transferred to tolerant animals. Whatever the tactics may be whereby transferred leucocytes from sensitized donors procure the abolition of tolerance, they probably establish permanent cell lineages, or clones, in their new hosts as evidenced by the consistent persistence of the transferred sensitivity for hundreds of days.

The findings obtained in the present study concerning the origin, distribution, and persistence of blood borne, immunologically activated cells in mice grafted with homologous skin are very similar to those obtained by Brent, Brown, and Medawar (2). They have studied a delayed dermal hypersensitivity reaction in donor guinea pigs procured by *local* adoptive transfer of cells from hosts immunized by means of skin homografts.

By neither method of revealing passively acquired skin homograft sensitivity have we been able to confirm the finding of Najarian and Feldman (29, 30), and of Kretschmer and Pérez-Tamayo (54) that transfer of immunologically activated lymphoid cells in Millipore chambers confers sensitivity upon

the host. Russell (55), too, has been unable to abolish tolerance in mice by transfer of cells in chambers. The reason for this discrepancy is at present obscure.

SUMMARY

Mice or rats that have been rendered tolerant of skin homografts from an alien donor strain furnish the basis of a very sensitive and objective test system for investigating the competence of cellular inocula from specifically immunized isologous donors to transfer sensitivity adoptively.

By means of this test system it has been shown that immunologically "activated" cells, capable of transferring homograft sensitivity, are present in the blood, peritoneal exudates, and regional nodes of animals that have rejected skin homografts. Leucocytes were as effective as regional node cells. Activated cells were first demonstrable in the regional nodes and blood of skin homograft recipients at the same time,—on the 6th postoperative day,—suggesting that these cells must enter the circulation very soon after their formation in the nodes. Moreover, when sensitization was effected by skin homografts, but not by means of splenic cell suspensions inoculated intraperitoneally, activated cells are highly persistent, still being demonstrable in both the blood and the nodes more than a year after sensitization.

The finding that thoracic duct cells, which are almost exclusively lymphocytes, were just as effective as leucocytes or regional nodes in transferring sensitivity in rats formally identifies the cell type responsible for transferring sensitivity in the various tissues tested.

Attempts to transfer sensitivity to homografts in normal mice or tolerant mice by means of larger dosages of activated lymphoid cells sequestered in Millipore chambers inserted intraperitoneally were unsuccessful.

All this, and other evidence presented, lends strength to the thesis that skin homograft immunity is a cell-mediated reaction.

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