THE SENSITIZATION OF RATS BY ALLOGRAFTS TRANSPLANTED TO ALYMPHATIC PEDICLES OF SKIN

By N. L. TILNEY,* M.D., and J. L. GOWANS, M.B.

(From the Medical Research Council, Cellular Immunology Unit, Sir William Dunn School of Pathology, Oxford University, Oxford, England)

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Allografts of skin are destroyed rapidly only if they establish lymphatic connections with the host (1); kidney allografts are destroyed even if such connections are prevented from forming (2). It has been suggested that the behavior of kidney grafts may be explained by a process of "peripheral sensitization" in which the graft antigens stimulate circulating lymphocytes as they pass through the vascular bed of the organ itself (3, 4). With orthotopic allografts of skin the requirement for lymphatics is usually taken to mean that sensitization occurs centrally in the regional lymph nodes after the arrival of antigen by way of the afferent lymphatics. Alternatively, lymphocytes might become sensititized as they migrate from the blood into the skin; the afferent lymphatics would then provide the route by which peripherally sensitized cells travel from the skin to the regional nodes. While the present evidence on skin grafts does not enable a choice to be made between these alternatives, the importance of lymphatics is not in dispute and their role in mediating sensitization is emphasized by the prolonged survival of grafts placed in certain immunologically privileged sites which do not provide an effective lymphatic drainage, for example, the hamster cheek pouch (5). The question then becomes, if kidneys can sensitize their hosts by way of the blood, why cannot fully vascularized grafts in immunologically privileged sites do the same? This problem has been examined by observing the fate of skin allografts placed in alymphatic skin pedicles raised on the backs of rats, following the technique of Barker and Billingham (1). The results show that the ablation of draining lymphatics does not fully protect a skin allograft in the rat and that sensitization of the host can occur eventually by another route.

Materials and Methods

Donor skin for allografts was taken from inbred albino (AO) rats. Recipients were either members of an inbred strain of hooded rat (HO), or an F_1 hybrid between this and the DA inbred strain. These three strains all differ from each other at the strong AgB locus. Auto-

* Present address: Department of Surgery, Peter Bent Brigham Hospital, Boston, Mass.

grafts were performed on rats belonging to a closed, noninbred albino colony and on (HO × DA)F_1 hybrids.

Full thickness skin grafts were prepared from the abdominal skin of donor animals and transferred to the lateral thoracic wall of recipients. Rejection was scored as the interval in days between transplantation and the appearance of gross necrosis of the skin surface.

Skin Pedicles.—The preparation in rats was a modification of the alymphatic skin pedicle employed in guinea pigs by Frey and Wenk (6), and later by Barker and Billingham (1).

The site chosen for the pedicle in the rat was a small segment of skin at the costovertebral angle, supplied solely by the subcostal artery. The lymphatic drainage of the area is illustrated in Fig. 1. A large lymphatic trunk runs cephalad in the deep dermis, and then courses deep to the panniculus carnosus to enter the middle two of the four axillary lymph nodes. A minor channel branches off and continues superficially to the panniculus in the deep dermis and enters the middle of three brachial nodes, high on the triceps muscle.

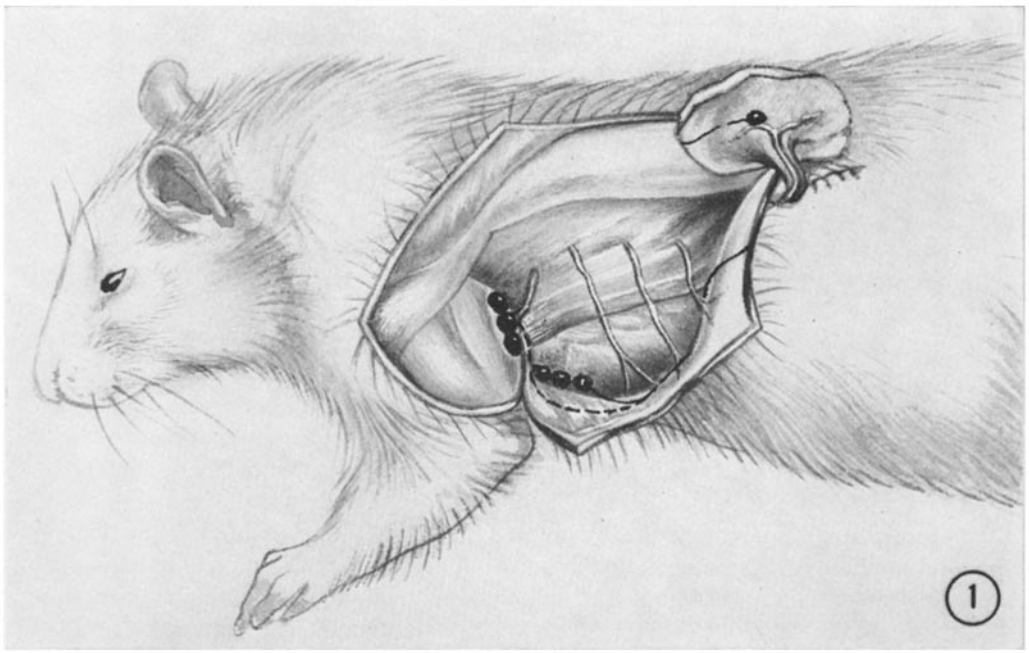


FIG. 1. A skin pedicle is isolated around its vascular stalk on the flank of a rat. The single lymphatic trunk from the pedicle divides into a major channel (solid line) which runs beneath the panniculus carnosus to the axillary lymph nodes, and a secondary channel (dotted line) which runs superficially to the panniculus and drains into the brachial lymph nodes.

Skin pedicles were raised under ether anesthesia on rats of either sex, weighing between 200 and 350 g. After clipping the fur and washing the skin with 1% Cetavlon (Imperial Chemical Industries, Ltd., Macclesfield, England), a circular incision was made through the panniculus to the underlying fascia. The neurovascular bundle supplying the pedicle was isolated from its surrounding areolar tissue as it emerged beneath the 13th rib. The skin defect was

closed with continuous sutures, leaving a small gap for the vascular stalk. A circular piece of 1.5 mm silicone rubber sheet (Esco Rubber Ltd., London, England), 2.5 cm in diameter, served as a backing for the isolated pedicle and was glued to the flank of the animal with methyl 2-cyanoacrylate adhesive (Ethicon, Inc., Somerville, N. J.). The vascular stalk was led by way of a radial slit through a hole 4 mm in diameter in the center of the rubber backing. The pedicle was positioned on the backing with two loosely applied sutures to prevent torsion. A full thickness section of skin was removed from the center of the pedicle, leaving the panniculus and subdermal vessels intact, and a carefully fitted, full thickness skin graft of standard size was placed in the defect and anchored with corner sutures. A group of pedicles without grafts was made for histologic comparison. Acriflavine-penicillin-streptomycin jelly was applied to all the cut surfaces and the pedicle was covered with sterile vaseline gauze. An adhesive strip was applied transversely to secure the preparation to the animal, whose trunk was then wrapped in several turns of gauze followed by Elastoplast bandage (Smith & Nephew Ltd., Hull, England).

Postoperative Course.—The animals were kept in separate cages to prevent them from chewing each other's bandages. Dressings were changed twice a week under ether anesthesia throughout the experimental period. The grafts healed normally into the pedicles which soon became thickened with lymphedema. Hair, together with a cuticular "ghost," could be lifted from the graft and surrounding pedicle within 3 wk. The subsequent feeble growth of hair was not considered a sign that either the grafts or the pedicles were in poor condition because the clipped hair on normal skin did not grow while covered by dressings.

The neurovascular bundle which supplied the pedicle remained thin with the nutrient vessel plainly visible. Histologic sections, stained with hematoxylin and eosin, showed the stalks to contain an artery, vein, and cutaneous nerves, loosely bound in areolar tissue. No lymphatic channels were seen. By 6 wk, a plexus of small venules had replaced the single subcostal vein, while marked fibroblastic proliferation was noted in the surrounding tissue.

About one-third of the pedicles survived for more than 3 wk. The usual termination was rapid infarction from kinking or torsion of the stalk. Infection, which occurred occasionally beneath the grafts, was presumably favored by the edema in the pedicles.

Lymphatic Regeneration.—0.05 ml volumes of either colloidal carbon (Pelikan ink, Günter-Wagner, Hanover, Germany) or ^{131}I-human serum albumin (Radiochemical Centre, Amersham, England, 20 mg albumin/ml) were injected intradermally into the grafted pedicles to identify regenerating lymphatics and the reestablishment of lymphatic drainage. Lymph nodes were then either inspected for carbon staining or teased apart and their radioactivity counted on a Packard gamma scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.).

Adoptive Immunization.—HO strain rats were sensitized with bilateral AO skin allografts transplanted to the lateral chest wall and boosted 9–11 days later with 10^8 pooled AO lymph node and spleen cells injected subcutaneously in four separate sites. Thoracic duct lymphocytes were collected from these donors by the method of Bollman, Cain, and Grindlay (7), 2 wk after boosting. Cells from the first 12 hr collection of lymph were washed and injected intravenously in varying doses into syngeneic recipients (8).

RESULTS

Survival of Skin Allografts on Alymphatic Pedicles of Skin.—Allografts were prepared with AO donors and (HO × DA) F_1 recipients while noninbred albinos or (HO × DA) F_1 rats were used for the autografts. First-set orthotopic allografts of skin were rejected between 9 and 13 days (Table I). Assessing the survival of grafts transplanted to skin pedicles was complicated by mechanical

hazards to the vascular stalk which led to the infarction of a considerable number of the preparations. In the allograft series, 39 rats were accumulated on which pedicles survived between 20 and 75 days, while a comparable series of 28 pedicles bearing autografts survived 20–52 days. Both autografts and allo-

TABLE I

Survival of Orthotopic Allografts of Skin Transplanted from AO to (HO × DA) F_1 Rats. Second-Set Grafts Transplanted 12–14 Days after a Single First-Set Graft.

	Days from transplantation to first sign of necrosis
1st SET	9, 10, 10, 10 10, 10, 11, 11 12, 12, 12, 13
2nd SET	5, 6, 6, 6 6, 6, 7, 7 8, 8

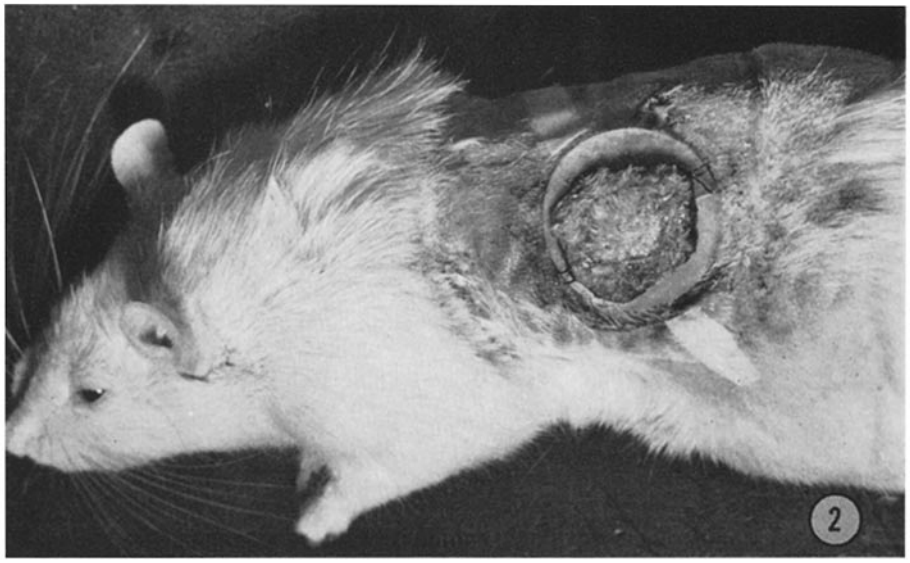
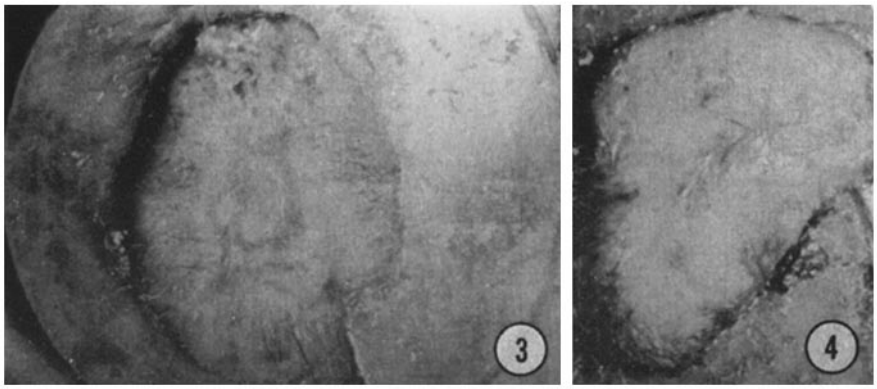


FIG. 2. Healthy autograft at 4 wk on an alymphatic skin pedicle. The pedicle rests on a circle of silicone rubber.

grafts contracted a little during the first 2 wk after transplantation. However, once healed, the autografts stabilized in size and were indistinguishable in texture from the surrounding pedicle skin (Fig. 2). In contrast, the allografts in pedicles, although surviving considerably longer than orthotopic allografts, con-

tinued to contract, became increasingly pale, and were reduced in several weeks to small scars (Figs. 3 and 4). The difference in surface area between autografts and allografts became obvious from the 3rd wk after placement (Fig. 5). Clearly, some type of chronic host response acted upon allografts in the skin pedicles.

Histologic sections of autografts and allografts, removed at weekly intervals, showed less striking differences. Mononuclear cells filled the graft bed and infiltrated the dermis of both preparations. Subdermal lymphatics were distended and packed with lymphocytes, and there was sporadic lymphocytic invasion of basal epidermal layers of autografts and allografts. The stratified layers of



FIGS. 3 and 4. Allografts 4 and 6 wk after implantation on skin pedicles. The graft at 4 wk has contracted considerably; only a scar remains at 6 wk. The pedicles have been drawn inwards by the contraction of the grafts.

autograft epithelial cells remained well defined with healthy nuclei, but the allograft epidermis became atrophic and showed pale, waxy epithelial cells with indistinct cell boundaries and irregular nuclei. Capillary growth was more exuberant in the autograft beds than in those of the allografts. The dermis and epidermis of pedicles without grafts were also infiltrated with mononuclear cells but less extensively than the grafted preparations. The axillary lymph nodes, which normally drain the area of intact skin from which the pedicles were prepared, showed no significant histological changes in either group.

Sensitization by Allografts on Skin Pedicles.—Rats were challenged with orthotopic skin allografts to determine whether the contracture of the allografts in pedicles was associated with sensitization of the host. Table I shows that normal (HO × DA) F_1 rats, which had been grafted orthotopically with AO skin 12–14 days previously, rejected second-set grafts in 5–8 days.

Healthy allografted pedicles, which had been in place from 7 to 67 days, were removed from 28 rats and skin allografts were transplanted to the opposite

chest walls. The tempo of graft rejection divided the animals into two distinct groups (Fig. 6). 13 rats, whose pedicles were removed before 23 days, rejected their test grafts with a first-set tempo. On the other hand, 15 animals with long-term pedicles rejected their allografts in an accelerated manner. There was no doubt that allografts on the skin pedicles eventually sensitized their hosts.

Lymphatic Regeneration in Skin Pedicles.—The results recorded in Fig. 6 made it crucial to determine whether regeneration of lymphatics along the

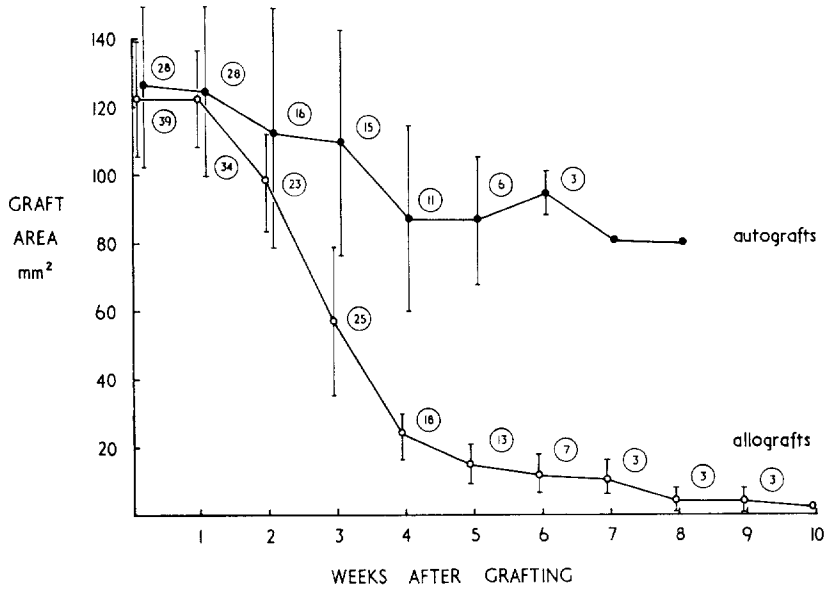


FIG. 5. The surface areas of autografts (noninbred albino or [HO × DA]F_1) and allografts (AO → [HO × DA]F_1) are compared at various times after transplantation to skin pedicles. Autografts change little in size but the allografts shrink progressively. Encircled figures refer to the number of animals providing measurements at each interval of time. The decrease in the number of animals with time is due to losses from infarction of the pedicles.

vascular stalks of the long-standing pedicles was responsible for the sensitization to orthotopic allografts observed in these animals.

The normal lymphatic drainage from the area of skin at the site of the pedicle runs in the deep dermis to the axillary and brachial lymph nodes and was readily demonstrated by an intradermal injection of carbon. A circular incision, corresponding in location and size to the skin pedicle, was sufficient to isolate completely the circle of skin from the draining lymphoid tissue if it merely divided the dermis but left the panniculus carnosus and subcutaneous tissues intact. After intradermal injection, carbon flowed forward with the dermal lymphatics to the cut edge of the skin and split from the transected lymph channels; no

staining was seen in any lymph nodes. Grafted pedicles on 15 animals, ranging from fresh preparations to those which had been established for 49 days, were injected intradermally with colloidal carbon. Again no lymph nodes became stained nor could any lymphatics be identified in the vascular stalk.

A more stringent test of lymphatic regeneration consisted of injecting 0.05 ml of ^{131}I-human serum albumin intradermally and assaying radioactivity in the

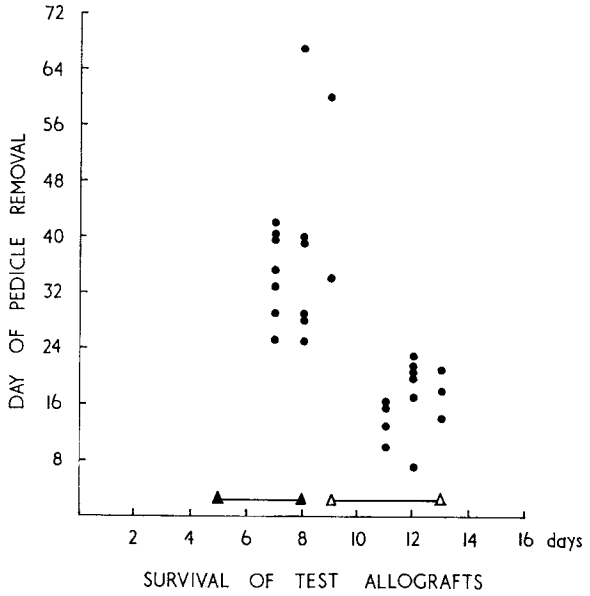


FIG. 6. Fate of orthotopic allografts of skin on rats which had borne allografted skin pedicles for varying periods of time (all allografts: AO → [HO × DA]F_1). Test grafts were destroyed with a first-set tempo when the grafted pedicles had been in place for up to about 3 wk. Beyond this time, test grafts were destroyed with a second-set tempo. Survival time of individual test grafts (●), range of survivals of normal first-set (△), and normal second-set (▲) allografts.

possible lymphoid drainage sites. After such an injection into normal skin, the uptake of radioactivity into the ipsilateral draining nodes reached a peak by 30 min and then declined gradually over 24 hr (Fig. 7). No other group of nodes showed significant radioactivity although small amounts accumulated slowly in the liver and spleen, presumably after leakage into the blood stream.

14 pedicles bearing either autografts or allografts, and ranging in age from 21 to 75 days, were injected intradermally with ^{131}I-human serum albumin. No radioactivity was detected in axillary, brachial, inguinal, renal, portal and superficial and deep parathymic lymph nodes which were removed between 1 and 48 hr after injection (Fig. 7). A few counts were detected in the liver and

spleen after many hours, presumably due to the direct entry of traces of labeled albumin into the blood vessels of the pedicle.

Susceptibility of Allografts on Skin Pedicles to Sensitized Lymphocytes.—The tempo of destruction of allografts on skin pedicles was extremely slow, yet the sensitivity to which they eventually gave rise was sufficient to mount a second-set reaction against orthotopic test grafts. Three experiments were carried out to determine if the pedicle grafts were relatively insensitive to the effector mechanism of the homograft reaction.

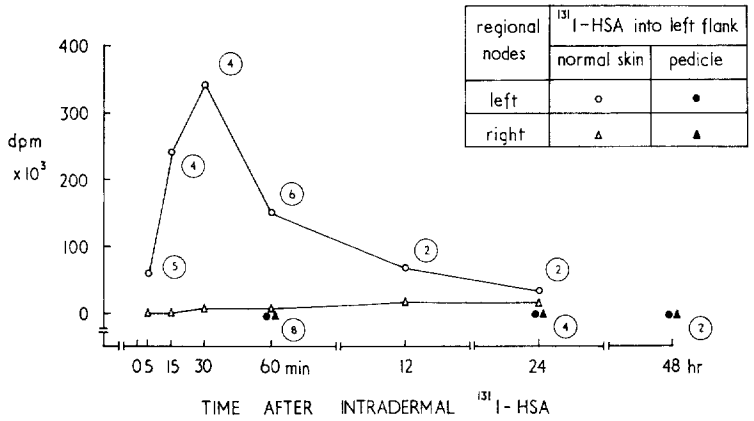


FIG. 7. Absence of lymphatic drainage from long-standing skin pedicles. Uptake by regional lymph nodes of ^{131}I-human serum albumin after intradermal injection into normal skin (○, △) and into skin pedicles (●, ▲) on the left flank. Mean radioactivity in left (○, ●) and right (△, ▲) brachial and axillary nodes in groups of rats killed at various times after injection Encircled figures represent number of rats per group. There is rapid uptake of radioactivity into ipsilateral nodes draining normal skin, but no uptake from skin pedicles. dpm, disintegrations per minute.

First, orthotopic skin allografts were placed on the chest wall of eight rats carrying pedicles with well healed allografts from the same donor strain. Both allografts were rejected on each rat by 14 days after orthotopic grafting. A second series of seven (HO × DA) F_1 rats was sensitized by orthotopic grafts of AO skin. 12–14 days later, allografts of AO skin were placed at the same time on freshly prepared pedicles and, orthotopically, on the opposite chest walls. Both grafts were rejected within 7 days, showing the usual tempo of a second-set response. The third experiment tested the ability of thoracic duct cells from sensitized donors to adoptively destroy allografts on skin pedicles. Suspensions of lymphocytes were prepared from the first 12 hr collection of lymph from HO rats immunized against AO tissues and were injected intravenously in various doses into HO rats with pedicles carrying well healed allografts of AO skin.

Large numbers of cells caused rapid rejection of the allografts, while doses of 25×10^6 cells and less were only partially effective (Table II). The first sign of rejection in these grafts was a segmental paleness followed by generalized pallor; necrosis of the grafts then occurred within a few days.

DISCUSSION

The present experiments, which follow very closely in design the studies of Barker and Billngham (1) on guinea pigs, were undertaken to assess the importance of lymphatics in the sensitization of rats to skin allografts. The incentive for the work stems from the observation that animals become sensitized

TABLE II

Adoptive Destruction of Allografts on Skin Pedicles by Thoracic Duct Lymphocytes from Sensitized Donors

Intravenous dose of lymphocytes* ($\times 10^6$)	No. of recipients with grafted pedicles	Survival of grafts after cell transfer
		(*days*)
200	3	6, 2 × 7
100	4	7, 2 × 8, 11
50	6	8, 9, 10, 2 × 11, 13
25	9	2 × 11, 12, 2 × 13, 15, 16, 2 × >30
10	6	12, 13, 2 × 18, 2 × >30

* Thoracic duct lymphocytes obtained from HO rats sensitized with bilateral AO skin allografts and boosted 9–11 days later with a subcutaneous injection of 10^8 AO lymphoid cells. Thoracic duct cannulated 2 wk after boosting. Recipients were HO rats with pedicles which had been grafted with AO skin about 1 wk previously.

by kidney allografts despite the absence of lymphatic connections between graft and host (2, 4). If the kidney can sensitize its host by way of the blood then it is not clear why fully vascularized grafts of other tissues should not also be able to do the same.

The experiments have shown that allografts of rat skin enjoy a prolonged survival if they are transplanted to pedicles of skin lacking lymphatic drainage. However, between 3 and 4 wk after grafting, a striking difference developed between autografts and allografts on the skin pedicles; whereas the autografts remained healthy in appearance and did not change appreciably in size, the allografts contracted progressively until little remained but a scar. This progressive contracture was associated with sensitization of the host so that orthotopic test allografts placed elsewhere on the animal were rejected with a second-set tempo. Particular care was taken to determine whether the slow rejection of the allografts on the skin pedicles was associated with a regrowth of lymphatics. The conventional method of injecting colored material intradermally failed to

reveal any lymphatic regeneration. This conclusion was strongly reinforced by a test of greater sensitivity which showed that no radioactive material reached the lymph nodes after an intradermal injection of ^{131}I-human serum albumin into long-standing skin pedicles. Thus, it is highly probable that the allografts sensitized their hosts by way of the blood and not by way of lymphatics.

This work in rats confirms the study of Barker and Billingham (1) on guinea pigs to the extent that the survival of allografts on alymphatic pedicles was certainly prolonged. However, our results appear to differ when the long-term fate of the allografts is considered; all the allografts on rat pedicles progressively diminished in size during the 1st month after implantation, whereas those in guinea pigs remained healthy for up to 32 days without any evidence of contracture. Possibly, guinea pigs and rats differ in the extent to which other mechanisms, not depending upon intact lymphatic supplies, can lead eventually to rejection of allografts. On the other hand it must be emphasized that, in both studies, many of the experiments were terminated by infarction of the pedicles from torsion or kinking of their vascular stalks. 19 of the 25 pedicles in guinea pigs recorded by Barker and Billingham (1) were lost between 20 and 32 days, and 3 of the remaining pedicles bore grafts which were destroyed between 33 and 54 days by a specific reaction. These results suggest that a significant proportion of allografts might eventually have been destroyed on guinea pig skin pedicles if a large enough number of them had been available for inspection at a later time.

The behavior of allografts on skin pedicles in the rat is very similar to the behavior of grafts placed in certain immunologically privileged sites which do not provide an effective lymphatic drainage, in that their survival in these situations is prolonged but not necessarily indefinite. Lance (9, 10) has shown that endocrine tissues implanted into the cerebral cortex of dogs become infiltrated with cells and are finally destroyed. Similarly, one-half of hamster cheek pouch allografts transplanted orthotopically in the strain combinations CB → MHA and LSH → CB were rejected by 30 days (5), and rejection was often characterized by a slow, progressive contracture of the kind seen on the rat pedicles. Allografts transplanted to the anterior chamber of the eye can also lead to sensitization, although in this case the growth of lymphatics has not been excluded (11). Some doubt has also been cast on the importance of lymphatics for sensitization during the induction of delayed hypersensitivity to simple chemicals, since Macher and Chase (12) have shown that the fraction of an intradermal injection of a skin-sensitizing chemical, which is essential for sensitization, is that which remains bound in the skin.

The slow sensitization of the host by skin allografts on alymphatic pedicles could be explained by the process of peripheral sensitization in which circulating small lymphocytes are stimulated by antigen within the graft itself, presumably on the vascular endothelium. Some support for a process of this kind was ob-

tained by Strober and Gowans (4) in experiments on renal allografts in rats and it has been dramatically demonstrated by Pedersen and Morris (13) for renal allografts in sheep. In the latter, transformed lymphocytes were found in the vascular bed of the kidney and many also emerged in the efferent lymph. However, the organ was destroyed even if the lymph-borne cells were diverted through a fistula, so that the whole immunological response could be initiated and effected within the kidney itself. These experiments in sheep make it important to reinvestigate whether it is cells or antigen that are carried to the regional nodes by the lymphatics draining an orthotopic allograft of skin.

Considerable numbers of lymphocytes accumulated within the skin of both allografts and autografts as a consequence of the interruption of the lymphatic drainage from the pedicles on rats. As pointed out by Barker and Billingham (1), it is surprising that a rapid local reaction does not ensue between the host lymphocytes and the skin allograft analogous to that seen in the normal lymphocyte transfer reaction (14) or, more recently, in the kidney allografts described by Pedersen and Morris (13). In the rat it remains possible that a slow local reaction of this kind was responsible, at least in part, for the atrophy of the allografts, although histological examination gave no evidence of the kind recorded by Pedersen and Morris.

A final point requiring explanation is the contrast between the slow demise of allografts on alymphatic pedicles and the accelerated destruction of orthotopic test grafts placed elsewhere upon the same animal. If the degree of sensitization was sufficient to destroy the test grafts with a second-set tempo, why was the rejection of the grafts on the pedicles so prolonged? A number of experiments showed that grafts on pedicles could respond promptly to both active and adoptive immunization so the difference in the behavior of grafts in the two situations may simply reflect the greater vulnerability of freshly implanted allografts in which healing is still incomplete. Possibly only a freshly implanted graft would be destroyed rapidly by the low level of sensitization raised by an alymphatic allograft, but a strong degree of sensitization would destroy both grafts with the same tempo.

SUMMARY

Pedicles of skin which lacked a lymphatic drainage were raised on the backs of rats in order to study the importance of afferent lymphatics in sensitization by skin allografts. Although allografts transplanted to the alymphatic pedicles enjoyed a prolonged survival, they contracted progressively from about 3 wk after transplantation and were reduced eventually to small scars. In contrast, autografts survived unchanged in size for the life-span of the pedicles which carried them. The slow contracture of the allografts was associated with sensitization of the host because test allografts applied orthotopically were destroyed with a second-set tempo. No regeneration of lymphatics from the long-

standing pedicles could be demonstrated, and it was concluded that sensitization had occurred eventually through the blood, presumably by the process of peripheral sensitization.

Allografts on skin pedicles could be destroyed rapidly by active or adoptive immunization, so it is probable that the level of sensitization to which they themselves gave rise was a low one. Although it is not disputed that afferent lymphatics are essential for the rapid destruction of skin allografts, it is clear that the absence of a lymphatic supply does not permanently exempt them from immunological attack in the rat.

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