THE ROLE OF AFFERENT LYMPHATICS IN THE REJECTION OF SKIN HOMOGRAFTS*

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Both cytological studies and those concerned with the adoptive transfer of transplantation immunity have demonstrated that the principal seats of specific reactivity to orthotopic skin homografts are the regional lymph nodes (1). On this basis it has been widely assumed that the lymphatic vessels draining the graft bed carry antigenic material to the nodes, thus constituting the afferent path of the immunologic reflex. An observation which has been widely construed as sustaining this premise is the partial or complete exemption from rejection enjoyed by small homografts implanted into vascularized sites, such as the brain, or the cheek pouch of the Syrian hamster, which lack a conventional lymphatic drainage (2). However, anatomical differences in the vascular beds of such grafts, as compared with those of orthotopic skin grafts, combined with the impossibility of serially inspecting intracranial grafts, render this sort of evidence inconclusive and it has recently been questioned by Lance (3). More compelling is Lambert et al.'s (4) evidence that temporary lymphatic discontinuity, established in rabbits' ears bearing skin homografts, both delays regional node response and prolongs graft survival by the time required—about 4 days—for demonstrable alternative lymphatic channels to develop.

It may also be pointed out that there is no evidence that afferent lymph draining a recently transplanted orthotopic skin homograft contains effective antigenic material. However, Hall (5) has shown that the lymph, draining areas bearing recently transplanted skin homografts, contains much cellular debris though both the origin and the antigenic status of this material is uncertain.

In the case of renal homografts, on the other hand, there is cogent evidence that the establishment of lymphatic connections with the host is *not* essential for evocation of sensitivity (6–9). Particularly telling is Strober and Gowans' (10) demonstration that if a homologous kidney is coupled extracorporeally to a rat's femoral artery and vein for a period of 5–12 hr, the animal subsequently develops a state of specific sensitivity. This occurs despite the absence of any direct contact or lymphatic connections between the host tissues and the alien organ. This finding has lent considerable strength to Medawar's (11, 12) concept of "peripheral sensitization" according to which immunologically competent small lymphocytes in the blood stream engage with fixed antigens in the graft itself, possibly at the level of its vascular endothelium, and then return to the host, probably via the regional lymphatics. On reaching the

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nodes, or other lymphoid tissues, they settle out and, by cytologic transformation and mitotic activity, generate the effector cell population that mediates the immune response.

The experiments described in this communication make use of vascularized, skin pedicles, whose draining lymphatics can be preserved, interrupted, and restored at will, as beds for "orthotopic" skin homografts (13). It will be shown that an intact lymphatic drainage in the graft bed is an essential prerequisite for sensitization of the host. Evidence bearing upon the manner in which sensitivity to skin homografts is evoked and put into effect will also be presented.

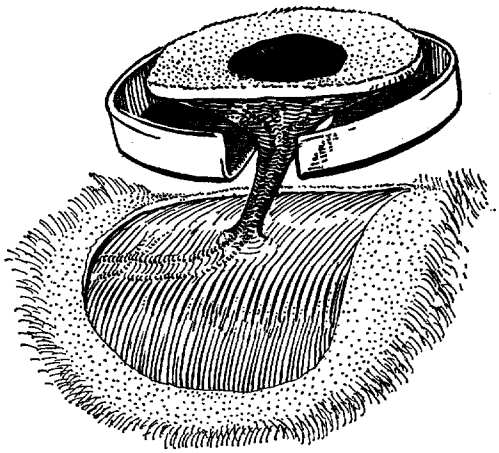

FIG. 1. Showing a freshly prepared isolated skin flap with its vascular umbilical cord and inlaid skin homograft. After closure of the skin defect with interrupted sutures the plastic dish housing the flap will be glued to the underlying skin.

Materials and Methods

The experimental animals were adult guinea pigs derived from domestically maintained sublines of the isogenic strains 2 and 13. These strains are known to differ by seven or eight transplantation antigens and reject mutually exchanged skin homografts within 10 days (14, 15).

Principle of the Experiments and Preparation of the Isolated Skin Flaps.—To evaluate the importance of lymphatic drainage for the rejection of orthotopic skin homografts it is essential to be able to observe the latter for long periods after transplantation to vascularized skin beds experimentally deprived of their draining lymphatic vessels. The procedure employed is a modification of that devised by Frey and Wenk (16).

Circular, full-thickness skin flaps, 3–4 cm in diameter and including the panniculus carnosus muscle, were cut from the shaved right flanks of guinea pigs anesthetized with Nembutal. A single vascular bundle or "umbilical cord" about 2–3 mm in diameter and containing an artery, a vein, and some nerves was left intact connecting the isolated flap with its host to maintain viability (Figs. 1 and 5). The circular skin defect was closed with interrupted sutures, leaving a small central gap for the emergent nourishing umbilical cord of the flap. The

latter was then placed in a small (35 × 10 mm) plastic Petri dish in the bottom of which a radial slit had been cut out to accommodate the umbilical cord (see Fig. 1). The dish was glued firmly to the underlying skin with Eastman 910 adhesive. After placing the lid on the Petri dish it was secured in place and protected from displacement by winding many turns of 1 inch wide plain gauze bandage round the animal's trunk, the under layers being affixed to the skin by means of Mastisol (17). Such a dressing allowed the animal normal mobility and the layer of bandage overlying the lid of the dish could easily be removed for periodic inspections without anesthetizing the animal (Fig. 2).

Immediately after their dissection, circular, shallow, partial thickness beds 1 cm in diameter were cut in the centers of the flaps. Thin, full-thickness skin grafts, removed from the donors' ears and freed from all adherent cartilage and fat, were then carefully fitted into these beds. The spotted coloration of the guinea pigs enabled pigmented skin grafts to be transplanted to white host skin flaps in all experiments so that epidermal melanocytes (18) could be used as donor epidermal markers to help follow the fate of grafts subjected to chronic reactions of low-grade intensity.

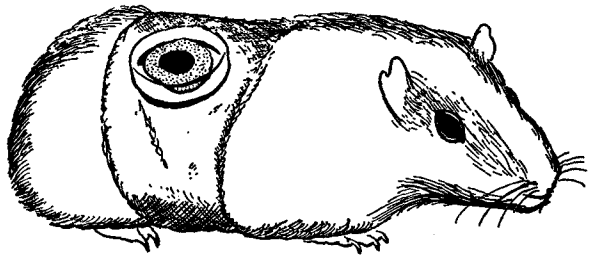

FIG. 2. A guinea pig bearing an intra-flap skin homograft in a plastic dish. Note the protective dressing applied around the animal's trunk.

Revelation of lymphatic vessels was accomplished by very superficial intradermal injections of a 2% aqueous solution of Patent Blue V, via a No. 30 gauge syringe needle (19). When injected into intact flank skin this dye promptly and consistently revealed the complex plexus of fine lymphatic vessels and the major afferent lymphatic channels leading to the prominent regional nodes which also became stained.

Skin grafting entailed the transfer of standard, full-thickness discs of ear skin, 1 cm in diameter and cut with the aid of a trephine, to partial thickness beds of appropriate size prepared in the skin flaps or on the sides of the hosts' chests. The operative technique has been described in full elsewhere (17).

Inspection and scoring of grafts: Primary inspection of all grafts was carried out on the 6th postoperative day. Subsequent inspections were made daily for the first 6–8 days and at less frequent intervals thereafter. The survival times of the grafts were determined on the basis of the outward appearance of their epidermis (17) and the median survival times estimated by Litchfield's (20) nomographic method. Representative grafts were excised at various intervals after transplantation, fixed in formol–mercuric chloride and embedded in paraffin wax. Transverse sections were cut at 8–10 μ and stained with Ehrlich's hematoxylin and eosin.

Sensitization of guinea pigs for transfer experiments was accomplished by grafting the animals bilaterally with 2.0–2.5-cm discs of the relatively thin skin from the donors' abdomens. This grafting was followed, 10–14 days later, by the intraperitoneal and subcutaneous administration of a coarse cell suspension in 4 ml of Hanks' solution corresponding to ¼ of a donor equiv-

alent of splenic and lymph node tissue. Animals were used for transfer experiments 8 days after receipt of the booster inoculations.

Suspensions of lymphoid cells were prepared by removing the donors' spleens, and axillary, brachial, cervical, inguinal, and mesenteric nodes aseptically, cutting them up into small fragments with scissors, and then lightly pressing them through a 50-mesh stainless steel screen. The expressed coarse fragments were taken up in Hanks' solution and gently sucked in and out of a pipette to effect further dissociation of the tissue. The resultant fine suspension was washed and resuspended in Hanks' solution.

Lymph node cell suspensions appropriate for intravenous injection were prepared according to our standard procedure (17).

Leukocytes were prepared by withdrawing blood from the heart into 20-ml syringes containing 5 IU of heparin and 2 ml of a 10% solution in normal saline of high molecular weight dextran. Erythrocytes were removed by sedimentation in the inverted syringe, with the needle up. The leukocyte-rich plasma was taken off and washed once in chilled Hanks' solution containing heparin (1 IU/ml). Leukocyte counts were made with the aid of a hemocytometer.

RESULTS

Base Line Data: Survival Times of Single Orthotopic Skin Homografts Exchanged between Guinea Pigs of Strains 2 and 13.—To provide controls for the work to be described, single ear skin grafts 1 cm in diameter were exchanged between 20 guinea pigs of strains 2 and 13, each graft being carefully fitted into a partial thickness bed of appropriate size prepared in the shaved skin of the lateral thoracic wall. With both the 13 → 2, donor/recipient strain combination and the converse one, all the grafts were rejected within 8–10 days (Table I). Because of the close similarity between the survival times of the two series of grafts (median survival times 8.7 ± 0.2 days and 9.2 ± 0.2 days, respectively), animals of both strains were used as donors or recipients, depending upon their availability.

Six animals which had rejected "first-set" grafts were rechallenged with donor strain skin 2–8 wk later. In all cases destruction of these grafts was complete on the 6th postoperative day.

The Fate of the Skin Flaps.—The pediculated skin flaps used to provide beds for the experimental skin homografts remained healthy and well vascularized as evidenced by the *uninterrupted* postoperative growth of their fur. It may be pointed out that in free skin grafts, by contrast, all the original hairs of the graft are shed in association with a phase of migratory and mitotic activity of the follicular epithelium which leads to cystic dilatation of the pilo-sebaceous units and total disruption of their normal structure. Follicular regeneration ensues and, usually after the 2nd postoperative week, new hairs begin to pierce the skin surface (21). The present finding therefore affords formal proof that it is temporary disruption of the skin's blood supply rather than massive trauma which is responsible for the phoenix-like activity of the follicles in free skin grafts.

On the 6th postoperative day the flaps were swollen by edema and there was build-up of granulation tissue on the cut surfaces. Subsequently, epithelium

migrated down from the upper surface of the flap resurfacing its edges and underside. The vascular pedicle slowly increased in thickness and ultimately became epithelialized, so that by about the 30th postoperative day the flap had assumed a somewhat mushroom-like shape with a pedicle of 5 mm or more in diameter. Although the rate of blood flow through these flaps is not known it was found that on severance of the umbilical cord a fine stream of blood under arterial pressure spurted out immediately.

TABLE I

Survival Times of First- and Second-Set Ear Skin Homografts Transplanted Orthotopically to Partial Thickness Beds Prepared in Intact Flank Skin, and of First-Set Skin Grafts Transplanted to Similar Beds Prepared in Standard Partially Isolated Skin Flaps in Guinea Pigs

Location of graft bed	Type of graft	No. of animals tested	Survival times of grafts	Median survival time
			days	*days*
Intact flank skin	First-set	10 (strain 13 → strain 2)	8 × 8; 2 × 9	8.7 ± 0.2
		10 (strain 2 → strain 13)	6 × 8; 2 × 9; 2 × 10	9.2 ± 0.2
	Second-set	6	6 × < 6	<6
Isolated skin flap	First-set	25	4 × 20; 21; 6 × 22 2 × 23; 24; 25; 26 27; 28; 32; 2 × 33* 52; 54*; > 57‡; 100‡	

Survival times without superscripts indicate grafts that died as a consequence of non-specific necrosis of their host flaps.

* Indicates grafts that were rejected as a consequence of a specific reaction.

‡ Indicates surviving homografts in flaps that were removed from their protective dishes and sewn back into place on the day indicated.

Repeated tests in which Patent Blue V dye was injected intradermally into freshly prepared skin flaps or those prepared 6–30 or more days beforehand revealed a rich lymphatic network *within* the flap. However, they consistently failed to reveal the existence of any lymphatic connections between flap and the host's trunk, and the regional nodes were never stained. Likewise no lymphatics were observed in histological sections of established vascular umbilical cords. Additional testimony of the absence of a lymphatic drainage in the flaps was the observation that a clear, lymph-like fluid containing about 500 lymphocytes/mm^3 usually exuded from the cut surfaces of flaps transected to obtain specimens for histology.

In most instances the viability of the flaps terminated abruptly after 20–30 days through ischemic necrosis caused by torsion, shearing, or strangulation of their umbilical cords. Occasionally it was possible to preserve flap viability for

as long as 50 days or more. Loosening of the attachment of the plastic capsule containing the flap to the host's skin was frequently responsible for its demise.

The Fate of Skin Homografts in Isolated Skin Flaps.—Skin homografts transplanted to beds in freshly prepared skin flaps healed in just as rapidly and as well as grafts transplanted to intact skin. At the primary inspection a cuticular "ghost" could be picked off the graft surface to reveal a hyperplastic superficial epidermis displaying pigment dilution. Within a few days the grafts reassumed their intense black color and a sparse crop of hairs, typical of ear skin, was usually regenerated by about the 20th day. Of 25 skin homografts transplanted to flap beds, 22 remained in a perfectly healthy, autograft-like condition (Figs. 5 and 6) either until they were removed electively for histological examination, or they succumbed as a consequence of flap necrosis taking place 20–100 days postoperatively (see Table I). Although most of the grafts in this series were 1 cm in diameter, grafts 2 cm in diameter gave equally good results, indicating that graft size is not critical.

Histological examination of excised grafts of 22 days' duration confirmed their healthy status. It did, however, reveal the existence of some diffuse and perivascularly concentrated mononuclear cells beneath the graft dermis (see Fig. 6).

Three of the 25 grafts studied did appear to succumb to specific chronic, low-grade reactions, manifested by loss of hair, dilution of pigmentation, contracture, and weakness of epithelial attachment, at 33, 33, and 54 days respectively. In these cases epithelialization of the vascular umbilical cord had taken place to produce a rather robust-looking pedicle of attachment (see Fig. 4) so that the possibility existed that lymphatic connections had been reestablished between the isolated skin flap and the host.

It need hardly be stressed that the survival times of all the homografts in the present series represent highly significant prolongations of the life expectancy of similar grafts transplanted to sites prepared in intact skin—by a factor of more than two.

Since 3/25 grafts were *specifically* rejected after more than 30 days, in subsequent experiments in which homografts in isolated flaps were used as indicators of sensitization, rejections taking place after 30 days could not be interpreted as reliable evidence of sensitization of the host by the particular procedure under investigation. This assumption follows since the lymphatic isolation of the flaps could not be absolutely relied upon after this time.

The Susceptibility of Skin Homografts in Skin Flaps to Transplantation Immunity.—Since the efferent pathway of the immunologic reflex is provided by the vasculature of the graft, it was anticipated that specific active sensitization of the host would curtail the survival of skin homografts transplanted to skin flaps. Confirmatory evidence for this has been forthcoming. Skin homografts placed in isolated flaps prepared in the flank skin of guinea pigs that had

previously rejected orthotopic skin grafts from the donor strain were consistently rejected just as swiftly as if they had been transplanted orthotopically (Table II). Furthermore, when normal guinea pigs received two skin homografts on the same occasion, one transplanted to intact skin on the thoracic wall and the other transplanted to a skin flap, *both* grafts underwent simultaneous rejection 7–8 days after operation.

Likewise the intradermal inoculation of as few as 10–20 million homologous leukocytes, distributed over five different sites, in the intact trunk skin of hosts which received intra-flap test skin homografts at the same time also procured the rejection of the grafts. Well-established intra-flap homografts were only

TABLE II

Effect of Sensitization of the Host on the Survival of Skin Homografts in Standard Skin Flaps

Means of sensitization	Day of administration of sensitizing material ± day of intra-flap grafting	No. of animals tested	Survival times of intra-flap skin homografts
			days
Single orthotopic skin homograft	−20–30 days	6	$6 \times < 6$
Single orthotopic skin homograft	0	4	$7, 3 \times 8$
150×10^6 homologous lymph node cells, intravenously	+7–10	3	$8, 2 \times 9$
$10–20 \times 10^6$ (strain 2 × strain 13)F_1 hybrid lymph node cells intradermally	0	5	7, 8, 9, 16, 24

slightly less vulnerable than recently transplanted grafts to the sensitization elicited in their hosts by proxy. Thus grafts of 7–10 days' standing were rejected in 8–9 days when their hosts were injected intravenously with a suspension of 150 million donor lymphoid cells in Hanks' solution (see Table II).

More compelling evidence that the afferent arc of the immunologic reflex is interrupted in the skin flaps was forthcoming from the following experiment. Four guinea pigs which had borne healthy intra-flap skin homografts for 18–28 days, and whose grafts had succumbed as a consequence of flap necrosis, were rechallenged with skin homografts from the original donor strain, the challenge grafts being placed in newly created flaps on the *left* (i.e. previously unoperated) sides of their trunks. All the secondary grafts healed in and survived in a perfectly healthy condition for as long as their host flaps remained viable, 18–28 days, again indicating the inability on the part of the previous intra-flap grafts to sensitize the recipients (Table III).

The observation that skin homografts transplanted orthotopically to animals bearing long-established intra-flap grafts from the same strain were rejected in a nonimmune manner (see Table III) also indicated that the chronic presence of the intra-flap grafts had failed to sensitize their hosts.

Evidence that Interruption of Lymphatic Vessels Constitutes the Break in the Afferent Pathway of the Immunological Reflex.—As already mentioned, all attempts to demonstrate the presence of patent lymphatics in the vascular pedicles of flaps were unsuccessful (16). However, if a bridge of undivided skin, 1–3 cm wide, was left at one place on the perimeter of an isolated flap to maintain continuity with the host's trunk skin, lymphatic communication between the flap and host skin across the bridge was consistently demonstrable by dye injection. Skin homografts placed in such flaps were consistently rejected within 8–11 days if the bridges were located caudally (Table IV).

TABLE III

Evidence of the Failure of Intra-Flap Skin Homografts to Sensitize Their Hosts

Location of second-set test skin homografts transplanted to animals which had previously born intra-flap skin homografts for 20 days	No. of animals tested	Survival times of second-set test grafts (days)
Orthotopic	8	3 × 8; 4 × 9; 10
Intra-flap, on left flank	4	18*; 20*; 26*; 28*

* Survival of these grafts terminated by flap necrosis and not by specific rejection.

However, if the bridges were located *dorsally* on the flap perimeter, the survival times of the grafts ranged from 10–17 days (Table IV). This significant disparity in the survival times of the two series of grafts correlates with anatomical evidence that on the flank the predominant lymphatic channels are in the cephalo-caudad axis rather than dorso-ventral (22). This correlation suggests that the lymphatic drainage from flaps with caudally located skin bridges is superior to that of flaps with dorsally located skin bridges.

Keller's (22) careful anatomical studies of the guinea pig's lymphatic system suggested that if skin flaps were raised from skin sites near to the axilla, instead of the flank, a large lymphatic vessel would accompany the group of blood vessels in the umbilical cord and lead directly to the axillary lymph node. Dye injection tests confirmed that this was indeed the case. When homografts were placed in flaps located in the axillary region, and in which the lymphatic drainage had purposefully been preserved, they were rejected within 9–17 days. However, if a ligature was tied around the lymphatic vessels in a pedicle (revealed by dye injection of the flap) the usual anomalous longevity of intra-flap skin homografts obtained.

Lymphatic continuity between isolated skin flaps bearing healthy skin

TABLE IV

Effect of Preservation or Restoration of Lymphatic Drainage on Fate of Freshly Transplanted or Established Skin Homografts in Skin Flaps, and Effect of Regional Lymphadenectomy on Fate of Orthotopic Skin Homografts

Location and type of host flap	Location of intact skin bridge on flap perimeter	No. of animals tested	Survival times of intra-flap skin homografts
			days
Flank, with skin bridge, 1–3 cm wide, housed in plastic dish	Caudally	5	8, 9, 2 × 10, 11
Flank, with skin bridge 1–3 cm wide, housed in plastic dish	Dorsally	6	10, 2 × 13, 15, 16, 17
Axilla, individual lymphatic channel identified and preserved; flap housed in plastic dish	None	6	2 × 9, 10, 11, 12, 17
Axilla, individual lymphatic channel identified and ligated; flap housed in plastic dish	None	2	28, 28
* Flank, isolated flap of 22–25 days' duration bearing healthy skin homografts removed from plastic dish and sewn back into fresh wound to restore lymphatic continuity	None	4	14, 16, 19, 23
* Flank, isolated flap of 100 days' duration bearing healthy graft removed from dish and sewn back into fresh wound to restore lymphatic continuity	None	1	28
Flank, isolated flap dissected and immediately sewn back into wound, instead of being placed in plastic dish	None	6	13, 3 × 18, 19, 20
Excision of cervical, axillary, brachial and inguinal nodes, followed by transplantation of orthotopic skin homograft ipsilaterally	—	4	13, 3 × 14

* In these experiments the survival times indicate the number of days the established intra-flap homografts lived *after* the flaps were sewn back into freshly prepared wounds.

homografts of long standing (22–25 days) and their hosts could easily be restored simply by removal of the protective Petri dishes, trimming off a very thin layer of skin from the margins and undersides of the flaps, and then suturing them back in place in freshly cut full-thickness wounds, in the hosts' flank skin, leaving the vascular pedicles intact. Subsequent dye injections confirmed the reestablishment of lymphatic continuity. Such "replaced" flaps soon lost their initial privileged status as indicated by the rejection within 14–23 days of skin homografts which had hitherto survived for periods from 22 to 100 days (in one case).

To determine whether this belated rejection, after reincorporation of the host flap in trunk skin, is simply a function of the time required for restoration of a lymphatic drainage rather than a manifestation of incomplete graft adaptation (23) or some other phenomenon, the following experiment was carried out on six guinea pigs. A skin flap with its intact vascular pedicle was raised in the usual manner and a skin homograft inserted in a shallow bed at its center. However, instead of placing the flap in a protective plastic dish it was *immediately* sutured back into the wound from which it was taken. Although both the flaps and their grafts healed in with normal promptitude, five of the six grafts survived for at least 18 days, instead of the 9–10 day expectation of survival of similar grafts transplanted to beds prepared in intact flank skin (see Table IV). This significant prolongation of graft survival suggested that reestablishment of lymphatic continuity between flap skin and its bed, or perhaps with host skin at its edges, may require as long as 3–10 days. It also suggests that neither antigenic material nor activated lymphocytes from the homografts escape in the material that exudes from the undersurface of the flaps.

Influence of Lymphadenectomy and the Length of the Afferent Lymphatic Pathway on Skin Homograft Survival Times.—Surgical excision of the regional nodes that normally drain the skin of the side of the trunk—the cervical, axillary, brachial, and inguinal nodes—did not impair the healing-in of orthotopic skin homografts transplanted immediately after lymphadenectomy but did prolong their survival times by 3–4 days (see Table IV). This is in accord with the findings of similar experiments carried out on mice and rabbits (24, 25). The failure of regional lymphadenectomy to have a more striking influence on the longevity of skin homografts is probably related to the speed with which a collateral lymphatic drainage opens up. These findings certainly emphasize that lymphatic interruption must be *complete* if the afferent arc of the immunologic reflex is to be broken (4).

Although it has been suggested that the anomalous longevity of homografts placed in the testes is a consequence of the remoteness of this organ from its nearest draining lymph node near the kidney (26), we have been unable to show that the length of the afferent lymphatic path affects the survival of orthotopic homografts of skin. Ear skin homografts of DA rat skin transplanted

to beds prepared near to the ends of the tails of Lewis hosts were rejected just as rapidly as similar grafts transplanted to the lateral thoracic wall.[1]

Influence of Attracting Host Mononuclear Cells into Graft-Bearing, Isolated Skin Flaps.—If sensitization to homografts does occur peripherally and the anomalous survival of the intra-flap grafts is attributable to the inability of locally "activated" small lymphocytes to return to the host, any artifice that would increase the number of mononuclear cells entering the graft might procure graft destruction. The latter might be accomplished either (*a*) by a local graft-versus-host type reaction (27) or (*b*) as a consequence of an effective number of the immunologically "primed" lymphocytes returning to the host by the intravenous route after peregrination through the parenchyma.

As a means of procuring the infiltration of intra-flap skin homografts by a "nonspecific" population of mononuclear cells, delayed hypersensitivity to dinitrochlorobenzene was induced in three guinea pigs. Skin homografts were then transplanted to standard skin flaps on these animals. On the 7th and again on the 15th postoperative day the entire flap surface, including that of the inlaid skin homografts, was challenged with a 0.1% solution of dinitrochlorobenzene in acetone. Although both challenges incited typical delayed inflammatory reactions, the associated infiltration of the grafts by mononuclear cells did not prejudice their survival, since the grafts lived for 33, 34, and 86 days.

Ability of Isolated Skin Flaps to Sustain Skin Heterografts.—Small scale trials were carried out to determine whether the skin flaps would provide a favorable environment for heterografts and, by implication, indicate the importance of lymphatic drainage for sensitization to grafts of this type. It was found that ear skin heterografts from AU strain mice, BN rats, and CB hamsters were all rejected within 8–10 days after transplantation to flap sites, whereas similar grafts transplanted to beds prepared in intact guinea pig's skin were invariably completely necrotic within 6 days. Evidently the flaps do provide a minor degree of protection to heterografts, though greatly inferior to that afforded to homografts. A similar state of affairs applies to the hamster's cheek pouch and probably to the brain. It may be that the release of powerful, soluble, species-specific antigenic material into the host's blood stream is largely responsible for inciting a host's rejection of heterografts.

Attempts to Extend the Isolated Flap Technique to Animals of Other Species.—Isolated, vascularized skin flaps without demonstrable lymphatic continuity with the host have been constructed in adult rats and shown to be capable of prolonging the survival of skin homografts to a significant degree. With the Lewis → DA donor/host strain combination, where the median survival time of skin homografts transplanted to conventional orthotopic sites is 7.3 ±

[1] Barker, C. F., and R. E. Billingham. 1968. Unpublished observations.

0.72 days, intra-flap skin homografts have lived for 14–20 days. However, because of the rat's great propensity to chew its dressings, reinforcement of the plain bandage securing the flap-containing Petri dishes to the flank with plaster impregnated bandage was obligatory. Even this artifice proved only partially satisfactory, insofar as most of the flaps and their grafts were lost through the host's gnawing.

Mice were unsatisfactory for flap experiments on the grounds that only the main cephalo-caudad blood vessels are large enough for use in umbilical cord construction to sustain isolated skin flaps and these are accompanied by lymphatics. With the CBA → A combination homografts inlaid in such flaps were rejected almost as rapidly as those transplanted to conventional sites in the integument.

Donawick[2] has raised relatively large skin flaps on the flanks of calves and maintained them in 100 × 15 mm diameter plastic Petri dishes. However, because of edema and infection they proved unsatisfactory as graft sites.

Among the laboratory mammals the guinea pig seems to be the animal of choice for these preparations on account of its relatively large size, shape, and docile behavior.

Some Applications of the Isolated Flap Technique

The fact that skin homografts sustained in skin flaps are easily accessible for inspection under conditions which preclude their sensitizing the hosts and yet leave them fully susceptible to a state of preexisting or subsequently incited sensitivity suggested their possible utility as a sensitive tool in transplantation immunology. In the sections that follow experiments are described which indicate some of the possible applications of this tool.

Use of Intra-Flap Skin Homografts as Indicators of Adoptive Immunization.— The capacity of lymphoid cells from specifically sensitized animals to transfer transplantation immunity adoptively between members of an inbred strain is demonstrable in two ways: (*a*) in terms of their ability to enable normal, isologous recipients to reject, in an accelerated manner, test skin homografts from donors of the alien strain in respect of which the donors of the transferred lymphoid cells have been sensitized (24) or (*b*) by their ability to procure the destruction of healthy, well-established skin homografts on hosts previously rendered specifically tolerant of the tissue antigens of the alien donor strain more rapidly than a similar number of lymphoid cells from a *normal*, isologous donor (28). Analysis of the immunological performance of the transferred cells in their new host is complicated in procedure (*a*) by the fact that the host itself soon responds to the antigens of the challenge graft, so that weak levels of sensitivity of transfer origin may escape detection. In procedure (*b*),

[2] Donawick, W. J. 1968. Personal communication.

which is exceedingly sensitive in terms of the small number of sensitized cells required to abolish tolerance, the chimeric status of the host seems to be important. Persisting donor-type cells—descendants of the original tolerance-conferring inoculum—provide an antigenic stimulus for the transferred sensitized cells additional to that furnished by the tolerated skin homograft and probably contribute to the sensitivity of the method. The intra-flap skin homograft in the guinea pig suggested itself as a useful challenge tissue for adoptive transfer experiments on the grounds that it was sustained by a completely normal host which was normally incapable of developing sensitivity against it—at least during a 30 day observation period.

When relatively large numbers of isologous lymphoid cells or peripheral blood leukocytes (1–2 donor equivalents or 140–325 $\times$ 10^6 cells respectively) from sensitized animals were transferred intraperitoneally, destruction of intra-flap test skin homografts transplanted within the range 3 days before to 7 days after cell transfer was consistently complete within 10 days or less. There was no evidence that adoptive immunization of hosts prior to or concomitant with test skin grafting was more effective than when transfer of the sensitized cells was delayed until the 5th or even the 7th day after grafting. Indeed, the data (Table V) suggest that the transferred cells accomplished more rapidly the destruction of grafts that were already healed-in at the time of transfer than grafts that were freshly transplanted.

To define the lower limit of the number of sensitized cells that must be transferred to procure graft rejection, peripheral blood leukocytes from sensitized animals were transferred, in dosages ranging from 20–75 million, to isologous hosts which received intra-flap test grafts on the *same* day. It was found that whereas 75 million cells consistently brought about rejection in 7–14 days and 50 million cells resulted in the rejection of four out of six intra-flap grafts within 16 days, dosages of 30 or 20 million leukocytes were ineffective, in the sense that they allowed graft survival for 30 days or more in animals whose flaps survived for this length of time (see Table V).

It must be conceded that, as in all adoptive transfer experiments using normal, as opposed to specifically tolerant hosts, the remote possibility exists that the sensitivity recorded by the intra-flap homografts was due to active immunization of the host by antigenic material, originating from the homografts used to sensitize the primary hosts and unavoidably carried over into the secondary hosts with the transferred lymphoid cells (24). To minimize this risk many experiments were carried out with peripheral blood leukocytes, predominantly lymphocytes, since antigenic material is less likely to be associated with them than with lymph node or splenic cell suspensions.

A small scale test was carried out to appraise the capacity of immune serum to prejudice the well-being of intra-flap skin homografts. One group of four animals received three intraperitoneal injections of 2.5 ml of antiserum on

alternate days commencing on the day of test skin grafting. Two other guinea pigs were similarly treated except that administration of the antiserum was delayed until the 7th day *after* skin grafting. In the first series of tests all

TABLE V

Survival Times of Test Skin Homografts in Skin Flaps on Guinea Pigs That Received Isologous Lymphoid Cells, Peripheral Blood Leukocytes, or Serum from Specifically Sensitized Donors

Material transferred from specifically sensitized isologous donors	Day of transfer ± day of test grafting	No. of hosts tested	Survival times of test skin grafts in flaps	Interval between lymphoid cell transfer and destruction of test skin homograft
			days	*days*
1–2 donor lymphoid equivalents	−3	2	7, 7	7
1 donor lymphoid equivalent	0	2	6, 6	6
or 200 × 10^6 leukocytes		2	6, 6	
1 donor lymphoid equivalent		3	8, 8, 9	~6
2 donor lymphoid equivalents	+2	3	7, 8, 8	
150 × 10^6 leukocytes		2	7, 8	
1 donor lymphoid equivalent	+5	4	8, 9, 9, 10	~4
or 325 × 10^6 leukocytes		2	9, 9	
1 donor lymphoid equivalent	+7	3	10, 10, 11	~3
200 × 10^6 leukocytes		1	10	
75 × 10^6 leukocytes	0	5	7, 2 × 8, 13, 14	~10
50 × 10^6 leukocytes	0	6	7, 12, 13, 16, 30, 34*	
30 × 10^6 leukocytes	0	2	28*, 30	
20 × 10^6 leukocytes	0	3	30, 32, 60	
Serum 2.5 ml i.p. on days 0, +2, and +4	—	4	23*, 36*, 55, 67*	
Serum 2.5 ml i.p. on days +7, +9, and +11	—	2	24, 43	

* Indicates grafts that succumbed as a consequence of ischemic necrosis of their host flaps.

grafts had conspicuously weak surfaces at the first postoperative inspection and in some there appeared to be progressive deterioration to the point of complete necrosis. However, all the grafts subsequently made a full recovery,

as evidenced by the regeneration of an intensely black superficial epidermis, and enjoyed long survival times before flap necrosis occurred. Nevertheless, prolongation of serum treatment might well have brought about the complete destruction of these grafts. Even in those animals whose grafts had healed-in *before* initiation of antiserum treatment there were indications that the latter exerted a definite though transient adverse effect, for their epidermis too underwent a phase of weakness of attachment to the underlying dermis.

The findings of these serum experiments show very clearly that in guinea pigs as in certain other species (29, 30), humoral factors, under some conditions, exert a demonstrable adverse effect on skin homografts. It need hardly be emphasized that the effect of immune serum in the present study was trivial compared with that of sensitized cells.

TABLE VI

Concomitant Transplantation of an Orthotopic Skin Homograft and Intra-Flap Indicator Skin Homograft Followed by Excision of the Sensitizing Grafts at Intervals to Determine Duration of Residence Necessary for Sensitization

Time sensitizing grafts in place before excision	No. of animals tested	Survival times of "indicator" skin grafts in flaps
days		
2	4	36, 42, 59, 93
3	5	17*, 18, 36, 54*, 69*
4	6	9, 9, 17, 40*, 42*, 50
5	2	20, 25
6	4	3 × 10, 17
Not excised	4	7, 3 × 8

* Indicates grafts which were destroyed nonspecifically as a consequence of host flap necrosis.

Determination of the Period of Residence Required for an Orthotopic Skin Homograft to Sensitize its Host.—Each of a series of guinea pigs received two ear skin homografts from the same donor. One of these grafts, the "indicator," was transplanted to a standard flap raised on the host's right flank; the other (the "sensitizing" graft) was transplanted to a shallow bed cut in intact skin on its left flank. After the sensitizing grafts had been in residence for periods of 2–6 days they were excised, together with generous amounts of contiguous host tissue, and the indicator grafts observed for signs of the development of sensitivity on the part of the host.

The results, summarized in Table VI, show that when the sensitizing grafts were in place for 3 days or less only one of the indicator grafts was rejected within 30 days. 4 days' residence of the sensitizing grafts caused definite "rejection crises" at 6–10 days in the indicator grafts as evidenced by epithelial weakness and 50% of them succumbed. However, the remainder recovered and enjoyed long survival times.

The presence of the sensitizing grafts for a minimum of 5–6 days seemed mandatory for the indicator grafts to be rejected within 30 days. Indeed, only when the sensitizing grafts had been in place for 6 days did the majority of the animals reject their indicator grafts as rapidly as ordinary orthotopic skin homografts. Other studies indicate that a skin homograft must be in residence for a minimum of 4 days in order to sensitize the host (31, 32).

The relatively long period for which an orthotopic skin homograft must remain in residence on a guinea pig to evoke sensitivity is surprising since nearly all guinea pigs develop sensitivity to topically applied simple chemicals, such as oxazolone, even if the painted skin sites are excised within 24 hr of the application of the sensitizer (33). This finding implies that either a sufficient amount of contact allergen becomes fixed to epidermal tissue and enough of the resultant complex finds its way into the lymphatic drainage (33), or a sufficient number of host lymphocytes undergo peripheral sensitization and return to the host within this time (34) for delayed hypersensitivity to develop. The disparity with respect to the time taken for sensitivity to develop in response to skin grafts on the one hand and the chemical sensitizers on the other hand might, of course, be more apparent than real, depending upon the sensitivity of the system used to detect altered host reactivity.

Significance of Reestablishment of Direct Lymphatic or Vascular Channels between a Skin Graft and its Bed for Sensitization of Host.—Some evidence suggests that the presence of a skin homograft on a freshly prepared bed may lead to sensitization of the host even if steps are taken to prevent healing-in of the graft. McKhann and Berrian (32) found that, in mice, if skin homografts were removed from their beds and replaced by fresh grafts from the same donor strain every 24 hr for 4 days, and then removed permanently, the hosts could later be shown to be sensitized.

Confirmation of this interesting observation has been obtained with the aid of the intra-flap grafting system. An intra-flap indicator skin homograft was placed on one side of a guinea pig's body and, at the same time, an orthotopic skin homograft from the same donor strain was transplanted to a bed prepared in intact skin on the opposite side of its body. These sensitizing grafts were subsequently removed daily from their beds and replaced with fresh ear skin homografts for a 4 day period after which no further homografts were applied. Of the three animals treated in this way two rejected their intra-flap grafts after 9 and 23 days, respectively, indicating the development of a weak level of sensitivity on the part of the host. The remaining animal's graft survived for 90 days, its demise resulting from trauma. A weakness of both the present as well as McKhann and Berrian's (32) experimental design is that it fails to exclude the possibility that the sensitivity induced by the transiently applied skin homografts was actually caused by donor leukocytes unavoidably carried over in the graft vasculature.

Encouraged by this small-scale confirmation of Berrian and McKhann's observation, we sought evidence whether the copious lymph or tissue exudate that normally escapes from the cut surfaces of recently prepared skin flaps bearing skin homografts contains antigenic material capable of sensitizing the host if allowed to seep into an open wound.

It is pertinent to mention here that, unlike blood vessels, severed lymphatics in a cutaneous wound may remain open for as long as 48 hr, and materials introduced into the wound pass directly into the lymphatics through their gaping ends (35).

Standard flaps were cut from flank skin in the usual manner and skin homografts fitted into shallow beds at their centers. However, instead of closing the donor wounds around the bases of the vascular pedicles, the skin flaps were replaced directly into the defects from which they had been cut. Protective dressings were then applied and removed daily when the skin flaps were carefully lifted up from their beds and replaced to prevent healing and establishment of direct lymphatic continuity with the host. This maneuver was repeated daily for 5 days. The flap was again lifted, and, after closure of the wound beneath it, inserted into a plastic Petri dish in the standard manner. That the fluid which seeped from the flap into the wound beneath did not contain antigenic material was suggested by the observation that in only one out of five such preparations was the intra-flap graft rejected within 30 days.

The negative results of this experiment suggested that the skin flaps either prevented the escape of antigenic material from the grafts, or, alternatively, did not allow the return to the host of peripherally sensitized lymphocytes, as a consequence of the lymphatic interruption. To investigate these possibilities, we transplanted skin homografts to standard flaps housed in plastic dishes. Then, after 1 or 2 wk, the intra-flap homografts were carefully excised, together with a narrow margin of host tissue. At the same time the flaps were removed from their protective capsules and sewn into freshly prepared wounds in the flanks, care being taken not to injure the vascular umbilical cords. To determine whether this artifice resulted in sensitization of the host, and therefore obtain evidence whether there had been residual antigenic material and (or) peripherally sensitized lymphocytes trapped within the connective tissue of the flap, we prepared a second flap on the hitherto unoperated left flank of each animal and transplanted an "indicator" skin homograft to it. This operation was carried out 3 wk after the original flap had been resutured into the host skin. In none of six animals treated in this way did rejection occur within 30 days.

The present findings are consistent with previous failures to sensitize rats by means of orthotopic split-skin homografts prevented from making direct contact with host tissue by a cell-impermeable Millipore membrane (36), or to sensitize mice by means of suspensions of homologous cells sequestered in Millipore diffusion chambers inserted intraperitoneally or subcutaneously.

DISCUSSION

On the basis of studies employing as graft sites partially isolated, vascularized skin flaps deprived of lymphatic connections with the host, it has been shown that, for elicitation of hypersensitivity to orthotopic skin homografts, as for the induction of hypersensitivity to simple chemicals (16), an intact afferent lymphatic drainage in the host skin is mandatory—i.e., lymphatics constitute the afferent pathway of the immunologic reflex. The susceptibility of intra-flap skin homografts to specific sensitization, acquired either actively or adoptively, is consistent with the well-founded belief that blood vessels constitute the efferent pathway of the immunological reflex.

Immunologically, the status of skin grafts transplanted to alymphatic skin flaps is comparable to that of small homografts implanted into the brain, the hamster's cheek pouch and possibly the anterior chamber of the eye, in the sense that none of these sites have demonstrable lymphatic connections with regional nodes. In the hamster, isografts of cheek pouch "skin" survive and maintain their tissue-specific characteristics if transplanted to the animal's trunk (2). If thin grafts of ordinary skin are transplanted to very shallow beds cut in the centers of well-established pouch skin grafts, they are usually exempted from the normal rejection process despite their vascularized status. This situation closely resembles that obtaining in the partially isolated skin flaps in guinea pigs since the host cheek pouch skin connective tissue seems to be devoid of lymphatics (2).[1] In these various privileged sites specific sensitivity on the part of the host is immediately effective. Other sites in which the reactivity of an unsensitized host to orthotopic skin homografts is diminished are the integument of limbs affected by Milroy's disease in the dog (37)[2]—a congenital anomaly due to anatomical shortcomings of the lymphatic drainage; and areas of the integument of cattle from which the lymph is drained off through a thoracic duct cannula, irradiated extracorporeally, and then returned to the circulation (38).

Various experiments have been described emphasizing the need for *complete* interruption of the lymphatic drainage in its bed to obtain a significant prolongation of survival of an orthotopic skin homograft. Indicative that the richness of the lymphatic drainage in its bed may affect the life expectancy of a skin homograft was the finding that retention of small skin bridges uniting skin flaps to host skin resulted in a normal rate of rejection of intra-flap homografts when the bridges were located caudally, whereas homograft survival was prolonged when the bridges were sited dorsally. These observations correlate with the anatomy of the lymphatic vessels draining the guinea pig's flank (22).

The rate of reestablishment of lymphatic drainage in skin grafts has been repeatedly investigated by various means (39–41). The results of two different

[3] Barker, C. F., R. E. Billingham, and D. Patterson. 1968. Unpublished observations.

experiments in the present study bear upon this problem. Firstly, when skin flaps bearing healthy, long-established skin homografts were surgically replaced in appropriate beds to allow restoration of lymphatic drainage, the mean subsequent survival time of the grafts was 18 days—compared with the 9 day median survival time of a primary orthotopic skin homograft. This finding indicates that the time required for reestablishment of a functional lymphatic drainage in a free skin graft cannot exceed about 9 days. An independent confirmation of this estimate derived from the observation that the mean survival time of skin homografts transplanted to skin flaps which were *immediately* replaced in their donor sites after preparation was also 18 days. On the basis of India ink injection studies Scothorne (41) estimated that the lymphatic drainage of full thickness grafts of ear skin on guinea pigs' ears was restored on the 5th postoperative day by anastomosis of host lymphatics in the graft bed with preexisting *intrinsic* lymphatics of the graft. The richer vascularity of ear skin as compared with that of the trunk may account, in part, for the difference between the two estimates.

So far as orthotopic homografts of skin are concerned, one of the most important unresolved questions is the identity of the putative agent(s), transmitted by the lymphatics of the bed, which initiates the formation of "effector" cells, and probably the synthesis of humoral isoantibodies, in the regional nodes. Until recently it was almost axiomatic that antigenic material was involved. Hall's (5) studies on the afferent lymph draining skin graft sites on the limbs of sheep indicated that cellular debris passes along this route. However, the alternative possibility that the inductive phase of the response to homografts may occur peripherally, as a result of some kind of interaction between blood-borne immunologically competent cells (small lymphocytes) and fixed antigen, possibly at the surfaces of vascular endothelial and other cells (11, 12), merits serious consideration. Indeed, Strober and Gowans' (10) experiments show that extracorporeal circulation of a rat's arterial blood through the vascular bed of an ex vivo renal homograft and its return via the venous circulation for as short a time as 5 hr result in sensitization of the animal.

The exemption from rejection enjoyed by homografts in partially isolated skin flaps, or in other privileged sites, is not necessarily inconsistent with the concept of peripheral sensitization or difficult to reconcile with Strober and Gowans' (10) observations. Firstly, in the case of a free skin graft, peripheral activation of host lymphocytes may take place principally in the graft *bed*, or in its parenchyma, rather than within its vasculature, and the activated cells pass down the draining lymphatics. Secondly, differences in the amount of endothelium in the skin graft's vasculature as compared with that of a whole organ graft and the effective "contact time" available for interaction of host lymphocytes with graft antigen may be important factors (10).

If peripheral sensitization at the intravascular level is assumed to be less

efficient in the case of small grafts, with limited endothelial surfaces available for lymphocyte contact, the well-established sensitizing potency of small orthotopic skin homografts (42) is difficult to explain. Facts pertinent to this question are (*a*) that although vascular connection between a graft and its bed is not mandatory, the presence of a patent lymphatic drainage in the bed is essential for sensitization (4), and (*b*) that a minimum period of about 4 days' contact between a homograft and its bed is required. One possibility is that antigenic material of graft origin, or immunologically "primed" lymphocytes, promotes sensitization more effectively if allowed to travel in high concentration along a lymphatic channel impinging upon a *single* draining node system, rather than being diluted in the systemic venous circulation before reaching lymphoid tissue. The well-known relative inefficiency of the intravenous route for eliciting transplantation immunity and the finding that regional lymphadenectomy prolonged the survival of skin homografts by 4–5 days lend some support to this interpretation.

Another factor to be taken into consideration is the extent of the tissue trauma associated with free skin grafting—which entails approximation of two cut surfaces. This situation is likely to be highly effective in attracting macrophages to the site. Since these cells play an important intermediary role in processing some kinds of antigen (1, 43) and they are abundant in the afferent lymph draining from skin grafted sites (4, 5), they may be important in the inductive phase of sensitivity to homografts.

If endocrine homografts survive in immunologically privileged sites for prolonged periods of time (23), or if renal homografts are chronically maintained in conventional sites by immunosuppressive procedures (44), they may progressively become less vulnerable to immunological attack. After a certain "critical" period they may be capable of surviving a degree of resistance to which they would have succumbed at an earlier stage. One explanation of this phenomenon of graft "adaptation" turns upon the possibility that a progressive, surreptitious replacement of stromal or vascular endothelial cells takes place in the graft (23). The present finding that skin homografts which had been protected in skin flaps for up to 100 days were rejected after restoration of lymphatic connections between the flaps and the host's trunk hints that skin homografts do not undergo adaptation.

On the basis of various experimental findings (45) it was conceivable that, during its residence in a skin flap, a skin homograft might continuously release antigenic material into the host's venous circulation leading to a state of tolerance. That this did not occur in this experimental situation was evidenced by the fact that animals bearing intra-flap homografts of 2–43 days' standing reacted with normal vigor against second-set grafts transplanted to conventional sites.

SUMMARY

Experiments have been carried out on guinea pigs of two isogenic strains to elucidate the role of afferent lymphatic vessels in the rejection of orthotopic skin homografts. Graft beds were prepared in partially isolated skin flaps with an intact sustaining vascular "umbilical cord" in which a lymphatic connection with the host could be retained or abolished at will.

In the absence of demonstrable lymphatic connections between flap and host, intra-flap homografts long outlived similar grafts transplanted to conventional sites in intact skin and, rather than being specifically rejected, died as a consequence of ischemic necrosis of the flap. When lymphatic drainage was retained, intra-flap homografts were rejected in the usual manner. Hosts of long-term intra-flap homografts did not develop sensitivity, as evidenced by the "first set" type rejection of subsequent test grafts, or by the long-term survival of a second skin graft transplanted to a new flap raised on the opposite side of the host's body.

Intra-flap skin homografts were rejected if (*a*) the hosts had been presensitized, (*b*) they were grafted concomitantly with a skin homograft placed in a conventional site, or inoculated with a suspension of donor lymphoid cells, or (*c*) if the lymphatic drainage was restored by reimplantation of the hitherto partially isolated flap to an appropriate vascular bed. These findings and others indicate that an intact lymphatic drainage in its bed is essential for an orthotopic skin homograft to sensitize its host.

Various experiments were carried out in which intra-flap homografts were used as "indicators" for the acquisition of specific active or adoptive immunity by their hosts.

By transplanting skin homografts to conventional beds concomitantly with intra-flap grafts and then excising the former at various intervals, it has been found that a graft must be in residence for a minimum period of 4 days to evoke the development of a detectable level of sensitivity in the host. Furthermore, by replacing either freshly prepared or long-term skin flaps bearing skin homografts in vascular beds on the trunk and determining the subsequent survival times of the homografts, evidence has been obtained suggesting that reestablishment of a functional lymphatic system in a free skin graft may take as long as 9 days.

Using intra-flap homografts as indicators of adoptive immunization of the host, we found that as few as 50×10^6 isologous peripheral blood leukocytes from a specifically sensitized animal will transfer an effective level of sensitivity. We also found that hyperimmune serum, in relatively large amount, exerts a weak but definite adverse effect upon either freshly or recently transplanted intra-flap grafts.

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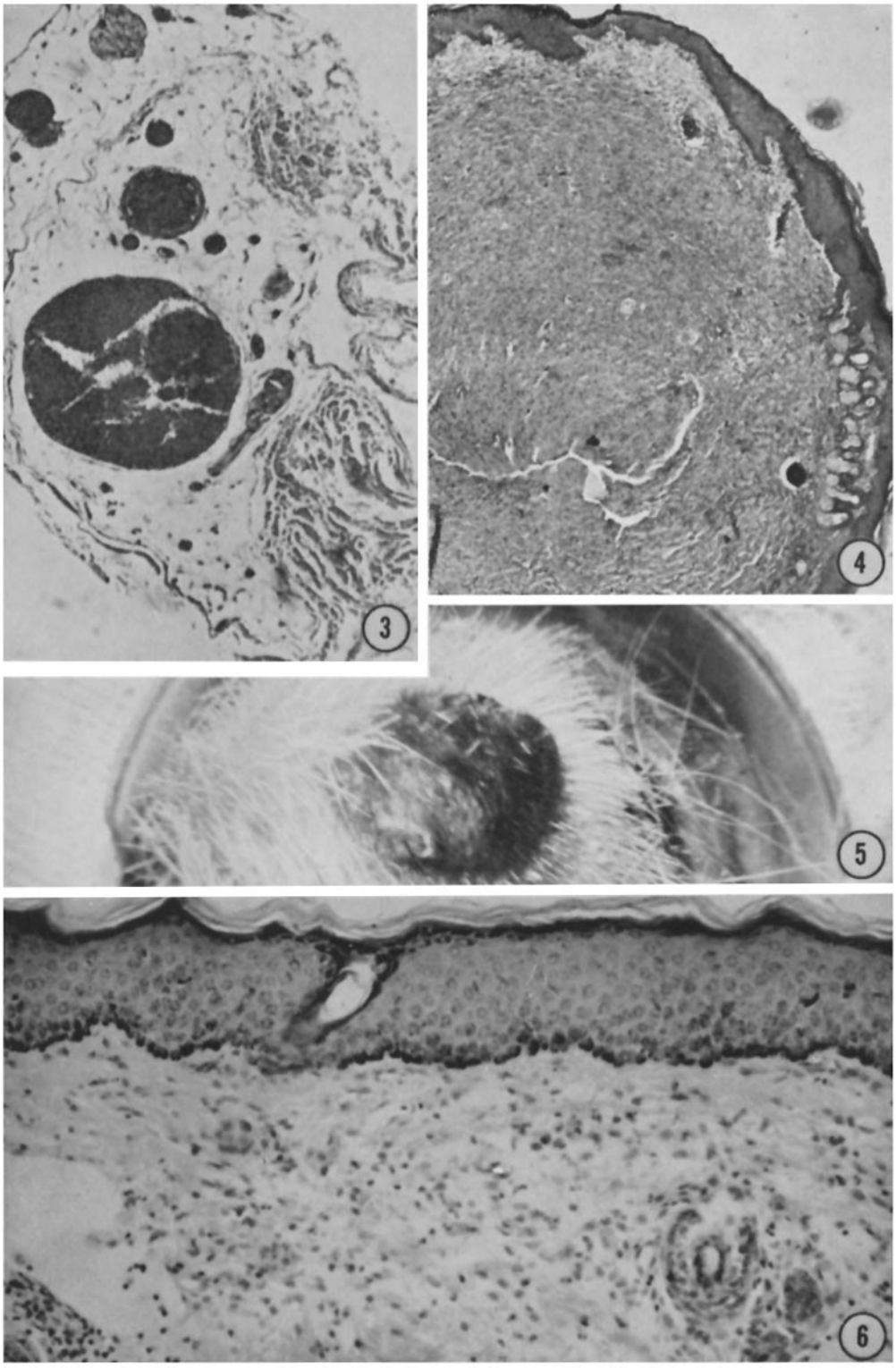
All histological specimens stained with hematoxylin and eosin.

FIG. 3. Transverse section through freshly prepared vascular "umbilical cord" uniting a skin flap to the site from which it was excised. Note the prominent blood vessels and nerve and the loosely knit connective tissue matrix that supports them. × 70.

FIG. 4. Section through vascular umbilical cord or pedicle sustaining a healthy, partially isolated skin flap preparation of 40 days' standing. This pedicle has become greatly thickened through the laying down of new fibrous connective tissue and it is completely epithelialized. Note the cluster of sebaceous glands of apparently de novo formation. × 35.

FIG. 5. An intra-flap pigmented ear skin homograft of 30 days' standing. The host flap has undergone some contraction and thickening so that it is now a fully epithelialized, mushroom-shaped structure. × 3.

FIG. 6. Section through pigmented ear skin homograft which has been in residence in a standard skin flap on an unsensitized guinea pig's trunk for 22 days. The healthy epidermis displays the characteristic stratigraphy of ear skin. There is a barely distinguishable mononuclear cell infiltration and two perivascular concentrations of mononuclear cells. × 220.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